THE ANTIBODY MOLECULE

by A. NISONOFF, J. E. HOPPER, and S. B. SPRING
A Volume in the MONOGRAPHS in IMMUNOLOGY Series

Most of our information concerning details of the properties and structure of the immunoglobulins has been acquired during the past fifteen years. This book summarizes this information and provides over 1200 references to the relevant literature. Perhaps no other book offers such complete and up-to-date descriptions of the immunoglobulins and their properties: the nature of the active site (size, specificity, structure, affinity labeling, reaction rates and equilibria); general properties of human immuno-globulins (IgG, IgM, IgA, IgD, IgE) and those of several other mammalian species; evolution of the immunoglobulins; amino acid sequences, allotypes, idiotypes and their interpretation in terms of the genetic control of antibody diversity; three-dimensional structure of immunoglobulins; interactions of heavy and light chains; and properties of homogeneous antibodies and of myeloma proteins with antibody activity.
1975, 560 pp., $34.50/£16.55

Revised Printing

CHEMICAL AND MOLECULAR BASIS OF NERVE ACTIVITY

by DAVID NACHMANSOHN
with
SUPPLEMENT I
Properties and Function of the Proteins of the Acetylcholine Cycle in Excitable Membranes

This revised edition contains a reprint of the 1959 edition, plus two new supplements. In the first one Dr. Nachmansohn outlines the highlights of the many important new developments in the biochemical investigations of nerve activity — particularly the properties and function of the specific proteins and enzymes associated with the

by DAVID NACHMANSOHN
and
SUPPLEMENT II
Towards a Molecular Model of Bioelectricity
by EBERHARD NEUMANN

action of acetylcholine. Supplement II by Dr. Eberhard Neumann describes in detail an exciting new development, an integral model of nerve excitability. The model integrates many electrophysiological, biochemical and thermodynamic data.
1975, 416 pp., $19.50/£9.35

METABOLISM OF SULFUR COMPOUNDS

Volume 7 of METABOLIC PATHWAYS, Third Edition
editor-in-chief: DAVID M. GREENBERG
CONTENTS: Y. Abiko, Metabolism of Coenzyme A. M. A. Eisenberg, Biotin. I. G. Leder, Thiamine, Biosynthesis and Function. M. Koike and K. Koike, Lipoic Acid. A. Meister, Biochemistry of Glutathione. W. C. Kenney, D. E. Edmond-son, and T. P. Singer, Covalent Adducts of Cysteine and Riboflavin. L. M. Siegel, Biochemistry of the Sulfur Cycle. R. H. De Meio, Sulfate Activation and Transfer. K. S. Dodgson and F. A. Rose, Sulfohydrolases. B. Sörbo, Thiosulfate Sulfurtransferase and Mercaptopyruvate Sulfur-transferase. M. Flavin, Methionine Biosynthesis. D. M. Green-berg, Biosynthesis of Cysteine and Cystine. D. M. Greenberg, Utilization and Dissimilation of Methionine. T. P. Singer, Oxidatve Metabolism of Cysteine and Cystine in Animal Tissues. J. D. Finkelstein, Enzyme Defects in Sulfur Amino Acid Metabolism in Man.
1975, 656 pp., $46.50/£22.30

INSECTS, SCIENCE, AND SOCIETY

(Proceedings of a Symposium on Insects, Science, and Society, held at Cornell University, Ithaca, New York, October 14-15, 1974)
edited by DAVID PIMENTEL
CONTENTS: **Insects and Their Social Implications:** J. J. McKelvey, Jr., Insects and Human Welfare. E. O. Wilson, The Evolutionary Significance of the Social Insects. **Communication among Insects:** R. D. Alexander, Natural Selection and Specialized Chorusing Behaviour in Acoustical Insects. W. L. Roelofs, Insect Communication — Chemical. **Patterns and Processes in Insect Interactions:** J. S. Kennedy, Insect Dispersal. M. D. Pathak, Utilization of Insect-Plant Inter-actions in Pest Control. **Insect Population Dynamics:** T. R. E. Southwood, The Dynamics of Insect Populations. P. S. Messenger, Parasites, Predators, and Population Dynamics. **Insect-Pest Management:** W. Klassen, Pest Management: Organization and Resources for Implementa-tion. L. D. Newsom, Pest Management: Concept to Practice.
1975, 310 pp., $15.00/£7.20

SYSTEMS ANALYSIS AND SIMULATION IN ECOLOGY,
VOLUME 3

edited by BERNARD C. PATTEN

This multi-volume work offers a comprehensive and authoritative picture of applications of systems science and computer technology to ecology. It shows how to model both small- and large-scale environmental systems, and provides numerous examples of applications ranging from dynamics of cell growth in culture to energy and material flows in terrestrial, freshwater, and marine ecosystems.
CONTENTS: **Part I. Ecosystem Modeling in the U.S. Inter-national Biological Program:** J. M. Neuhold, Introduction to Modeling in the Biomes. G. S. Innis, Role of Total Systems Models in the Grassland Biome Study. R. V. O'Neill, Modeling in the Eastern Deciduous Forest Biome. D. W. Goodall, Ecosystem Modeling in the Desert Biome. P. C. Miller, B. D. Collier, and F. L. Bunnell, Development of Ecosystem Modeling in the Tundra Biome. W. S. Overton, The Ecosystem Modeling Approach in the Coniferous Forest Biome. K. E. F. Watt, Critique and Comparison of Biome Ecosystem Modeling. **Part II. Models of Freshwater-Estuarine Ecosystems:** R. G. Wiegert, Simulation Modeling of the Algal-Fly Components of: a Thermal Ecosystem Effects of Spatial Heterogeneity, Time Delays, and Model Condensation. R. H. Boling, Jr., R. C. Petersen, and K. W. Cummins, Ecosystem Modeling for Small Woodland Streams. B. C. Patten, D. A. Egloff, and T. H. Richardson, Total Ecosystem Model for a Cove in Lake Texoma. D. M. Di Toro, et. al., Phytoplankton-Zooplankton-Nutrient Inter-action Model for Western Lake Erie. C. W. Chen and G. T. Orlob, Ecologic Simulation for Aquatic Environments.

1975, 622 pp., $39.50/£18.95

Cover picture
The globular star cluster Kron 3.
Some clusters may contain massive
black holes; see page 23.
(Anglo-Australian Telescope Board.)

nature

Volume 256   July 3, 1975

Guide to authors

● Articles are up to 3,000 words in length with at most six displayed items (figures and tables) and may either be reports of major research developments in a subject or broader reviews of progress.

● Letters are brief reports of research of unusual and wide interest, not in general longer than 1,000 words; at most they have three or four displayed items (figures and tables).

● 'Matters Arising' permits occasional short discussion of papers that have previously appeared in *Nature*. There is a limit of 300 words.

Manuscripts may be submitted either to London or Washington. Three typed copies should be submitted, each including lettered copies of figures. Typing (including references) should be double spaced. The title should be brief and informative. Pages should be numbered. References, tables and figure legends should start on separate pages. Experimental detail vital to the paper yet which would interrupt the narrative is best placed in the figure legends. Units should conform to the Système International. Greek characters should be identified in the margin on their first appearance. Equations should occupy single lines if possible, exp (a) is preferred to e^a if 'a' is more than one character. Articles should be accompanied by an abstract of not more than fifty words listing the main conclusions.

References are indicated by super-scripts in the text. See any contemporary *Nature* for style, but note:

(i) To refer to several references by the same author at once, only one reference number need be given.

(ii) Cite first and last pages.

Abbreviations should follow the *World List of Scientific Periodicals*, fourth ed. (Butterworth, 1963-65). Symposia are often difficult to refer to and only published or soon-to-be-published volumes should be mentioned in references. Their publisher and place of publication should be clearly indicated. 'Personal communication' and 'unpublished work' should be incorporated in the text.

Artwork should be sent with the manuscript. All artwork should be marked with the author's name. Line drawings should preferably be in Indian ink on heavy cartridge paper, although other materials are acceptable; thin, shiny, folded, torn or heavily handled material should be avoided. Matt rather than glossy photographs are preferred. Figures are usually reduced to one column width. The originals should be about as wide as a page of *Nature*. Figures, particularly maps, should contain nothing but essential material. It is preferred that the original be unlabelled, but with a copy containing lettering. Labelling on photographs should if possible be avoided entirely. We are always glad to see artwork for possible use on the cover, whether or not it is associated with a manuscript, but cannot guarantee the return of material.

In order to save on postal expenses we return only the top copy and artwork of manuscripts that we cannot publish.

A fuller guide appeared in *Nature* (246, 238; 1973).

Editor
David Davies

Deputy Editor
Roger Woodham

Editorial Staff
Gillian Boucher   Peter Newmark
John Gribbin   Colin Norman*
Sandy Grimwade   Allan Piper
John Hall   Miranda Robertson
Eleanor Lawrence   Fiona Selkirk
*Mary Lindley   Robert Vickers
Peter Milford   Mary Wade*
*Washington office

Publishing Director
Jenny Hughes

Cover picture

The lacewing fly.
Photography by Stephen Dalton.

Volume 256   July 10, 1975

Cover picture

The Princess and the Pea. An illustration by Edmond Dulac in a World War I Picture Book for the French Red Cross
See page 203.
Mary Evans Picture Library

nature

Guide to authors

● Articles are up to 3,000 words in length with at most six displayed·items (figures and tables) and may either be reports of major research developments in a subject or broader reviews of progress.

● Letters are brief reports of research of unusual and wide interest, not in general longer than 1,000 words; at most they have three or four displayed items (figures and tables).

● 'Matters Arising' permits occasional short discussion of papers that have previously appeared in *Nature*. There is a limit of 300 words.

Manuscripts may be submitted either to London or Washington. Three typed copies should be submitted, each including lettered copies of figures. Typing (including references) should be double spaced. The title should be brief and informative. Pages should be numbered. References, tables and figure legends should start on separate pages. Experimental detail vital to the paper yet which would interrupt the narrative is best placed in the figure legends. Units should conform to the Système International. Greek characters should be identified in the margin on their first appearance. Equations should occupy single lines if possible, exp (*a*) is preferred to e^a if '*a*' is more than one character. Articles should be accompanied by an abstract of not more than fifty words listing the main conclusions.

References are indicated by superscripts in the text. See any contemporary *Nature* for style, but note:

(i) To refer to several references by the same author at once, only one reference number need be given.

(ii) Cite first and last pages.

Abbreviations should follow the *World List of Scientific Periodicals*, fourth ed. (Butterworth, 1963-65). Symposia are often difficult to refer to and only published or soon-to-be-published volumes should be mentioned in references. Their publisher and place of publication should be clearly indicated. 'Personal communication' and 'unpublished work' should be incorporated in the text.

Artwork should be sent with the manuscript. All artwork should be marked with the author's name. Line drawings should preferably be in Indian ink on heavy cartridge paper, although other materials are acceptable; thin, shiny, folded, torn or heavily handled material should be avoided. Matt rather than glossy photographs are preferred. Figures are usually reduced to one column width. The originals should be about as wide as a page of *Nature*. Figures, particularly maps, should contain nothing but essential material. It is preferred that the original be unlabelled, but with a copy containing lettering. Labelling on photographs should if possible be avoided entirely. We are always glad to see artwork for possible use on the cover, whether or not it is associated with a manuscript, but cannot guarantee the return of material.

In order to save on postal expenses we return only the top copy and artwork of manuscripts that we cannot publish.

A fuller guide appeared in *Nature* (**246**, 238; 1973).

nature SUBSCRIPTION ORDER FORM

Volume 256   July 24, 1975

Guide to authors

● Articles are up to 3,000 words in length with at most six displayed items (figures and tables) and may either be reports of major research developments in a subject or broader reviews of progress.

● Letters are brief reports of research of unusual and wide interest, not in general longer than 1,000 words; at most they have three or four displayed items (figures and tables).

● 'Matters Arising' permits occasional short discussion of papers that have previously appeared in *Nature*. There is a limit of 300 words.

Manuscripts may be submitted either to London or Washington. Three typed copies should be submitted, each including lettered copies of figures. Typing (including references) should be double spaced. The title should be brief and informative. Pages should be numbered. References, tables and figure legends should start on separate pages. Experimental detail vital to the paper yet which would interrupt the narrative is best placed in the figure legends. Units should conform to the Système International. Greek characters should be identified in the margin on their first appearance. Equations should occupy single lines if possible, exp (a) is preferred to e^a if 'a' is more than one character. Articles should be accompanied by an abstract of not more than fifty words listing the main conclusions.

References are indicated by super-scripts in the text. See any contemporary *Nature* for style, but note:

(i) To refer to several references by the same author at once, only one reference number need be given.

(ii) Cite first and last pages.

Abbreviations should follow the *World List of Scientific Periodicals*, fourth ed. (Butterworth, 1963-65). Symposia are often difficult to refer to and only published or soon-to-be-published volumes should be mentioned in references. Their publisher and place of publication should be clearly indicated. 'Personal communication' and 'unpublished work' should be incorporated in the text.

Artwork should be sent with the manuscript All artwork should be marked with the author's name. Line drawings should preferably be in Indian ink on heavy cartridge paper, although other materials are acceptable; thin, shiny, folded, torn or heavily handled material should be avoided. Matt rather than glossy photographs are preferred. Figures are usually reduced to one column width. The originals should be about as wide as a page of *Nature*. Figures, particularly maps, should contain nothing but essential material. It is preferred that the original be unlabelled, but with a copy containing lettering. Labelling on photographs should if possible be avoided entirely. We are always glad to see artwork for possible use on the cover, whether or not it is associated with a manuscript, but cannot guarantee the return of material.

In order to save on postal expenses we return only the top copy and artwork of manuscripts that we cannot publish.

A fuller guide appeared in *Nature* (**246**, 238; 1973).

Editor
David Davies

Deputy Editor
Roger Woodham

Editorial Staff
Gillian Boucher   Peter Newmark
John Gribbin   Colin Norman*
*Sandy Grimwade   Allan Piper
John Hall'   Miranda Robertson
Eleanor Lawrence   Fiona Selkirk
Mary Lindley   Hilary Taphouse
Peter Milford   Robert Vickers
Mary Wade*
*Washington office

Publishing Director
Jenny Hughes

Cover picture

Primitive plant, *Cycas circinalis;*
see page 364.

Photograph by E. Arthur Bell

Guide to authors

● Articles are up to 3,000 words in length with at most six displayed items (figures and tables) and may either be reports of major research developments in a subject or broader reviews of progress.

● Letters are brief reports of research of unusual and wide interest, not in general longer than 1,000 words; at most they have three or four displayed items (figures and tables).

● 'Matters Arising' permits occasional short discussion of papers that have previously appeared in *Nature*. There is a limit of 300 words.

Manuscripts may be submitted either to London or Washington. Three typed copies should be submitted, each including lettered copies of figures. Typing (including references) should be double spaced. The title should be brief and informative. Pages should be numbered. References, tables and figure legends should start on separate pages. Experimental detail vital to the paper yet which would interrupt the narrative is best placed in the figure legends. Units should conform to the Système International. Greek characters should be identified in the margin on their first appearance. Equations should occupy single lines if possible, exp (a) is preferred to e^a if 'a' is more than one character Articles should be accompanied by an abstract of not more than fifty words listing the main conclusions.

References are indicated by superscripts in the text. See any contemporary *Nature* for style, but note:

(i) To refer to several references by the same author at once, only one reference number need be given.

(ii) Cite first and last pages.

Abbreviations should follow the *World List of Scientific Periodicals*, fourth ed. (Butterworth, 1963-65). Symposia are often difficult to refer to and only published or soon-to-be-published volumes should be mentioned in references. Their publisher and place of publication should be clearly indicated. 'Personal communication' and 'unpublished work' should be incorporated in the text.

Artwork should be sent with the manuscript. All artwork should be marked with the author's name. Line drawings should preferably be in Indian ink on heavy cartridge paper, although other materials are acceptable; thin, shiny, folded, torn or heavily handled material should be avoided. Matt rather than glossy photographs are preferred. Figures are usually reduced to one column width. The originals should be about as wide as a page of *Nature*. Figures, particularly maps, should contain nothing but essential material. It is preferred that the original be unlabelled, but with a copy containing lettering. Labelling on photographs should if possible be avoided entirely. We are always glad to see artwork for possible use on the cover, whether or not it is associated with a manuscript, but cannot guarantee the return of material.

In order to save on postal expenses we return only the top copy and artwork of manuscripts that we cannot publish.

A fuller guide appeared in *Nature* (246, 238; 1973).

Cover picture

Desert locusts settle for the night.
See pages 484, 486.
Photo: Jean Manuel, FAO Rome.

nature

Volume 256   August 7 1975

Guide to authors

● Articles are up to 3,000 words in length with at most six displayed items (figures and tables) and may either be reports of major research developments in a subject or broader reviews of progress.

● Letters are brief reports of research of unusual and wide interest, not in general longer than 1,000 words; at most they have three or four displayed items (figures and tables).

● 'Matters Arising' permits occasional short discussion of papers that have previously appeared in *Nature*. There is a limit of 300 words.

Manuscripts may be submitted either to London or Washington. Three typed copies should be submitted, each including lettered copies of figures. Typing (including references) should be double spaced. The title should be brief and informative. Pages should be numbered. References, tables and figure legends should start on separate pages. Experimental detail vital to the paper yet which would interrupt the narrative is best placed in the figure legends. Units should conform to the Système International. Greek characters should be identified in the margin on their first appearance. Equations should occupy single lines if possible, exp (a) is preferred to e^a if 'a' is more than one character. Articles should be accompanied by an abstract of not more than fifty words listing the main conclusions.

References are indicated by superscripts in the text. See any contemporary *Nature* for style, but note:

(i) To refer to several references by the same author at once, only one reference number need be given.

(ii) Cite first and last pages.

Abbreviations should follow the *World List of Scientific Periodicals*, fourth ed. (Butterworth, 1963-65). Symposia are often difficult to refer to and only published or soon-to-be-published volumes should be mentioned in references. Their publisher and place of publication should be clearly indicated. 'Personal communication' and 'unpublished work' should be incorporated in the text.

Artwork should be sent with the manuscript. All artwork should be marked with the author's name. Line drawings should preferably be in Indian ink on heavy cartridge paper, although other materials are acceptable; thin, shiny, folded, torn or heavily handled material should be avoided. Matt rather than glossy photographs are preferred. Figures are usually reduced to one column width. The originals should be about as wide as a page of *Nature*. Figures, particularly maps, should contain nothing but essential material. It is preferred that the original be unlabelled, but with a copy containing lettering. Labelling on photographs should if possible be avoided entirely. We are always glad to see artwork for possible use on the cover, whether or not it is associated with a manuscript, but cannot guarantee the return of material.

In order to save on postal expenses we return only the top copy and artwork of manuscripts that we cannot publish.

A fuller guide appeared in *Nature* (**246**, 238; 1973).

Cover picture
An idea Wheatstone missed.
For the ones he didn't, see page 533.
(Picture by Michael Freeman)

Volume 256   August 14 1975

Guide to authors

● Articles are up to 3,000 words in length with at most six displayed items (figures and tables) and may either be reports of major research developments in a subject or broader reviews of progress.

● Letters are brief reports of research of unusual and wide interest, not in general longer than 1,000 words; at most they have three or four displayed items (figures and tables).

● 'Matters Arising' permits occasional short discussion of papers that have previously appeared in *Nature*. There is a limit of 300 words.

Manuscripts may be submitted either to London or Washington. Three typed copies should be submitted, each including lettered copies of figures. Typing (including references) should be double spaced. The title should be brief and informative. Pages should be numbered. References, tables and figure legends should start on separate pages. Experimental detail vital to the paper yet which would interrupt the narrative is best placed in the figure legends. Units should conform to the Système International. Greek characters should be identified in the margin on their first appearance. Equations should occupy single lines if possible, exp (a) is preferred to e^a if 'a' is more than one character. Articles should be accompanied by an abstract of not more than fifty words listing the main conclusions.

References are indicated by superscripts in the text. See any contemporary *Nature* for style, but note:

(i) To refer to several references by the same author at once, only one reference number need be given.

(ii) Cite first and last pages.

Abbreviations should follow the *World List of Scientific Periodicals*, fourth ed. (Butterworth, 1963-65). Symposia are often difficult to refer to and only published or soon-to-be-published volumes should be mentioned in references. Their publisher and place of publication should be clearly indicated. 'Personal communication' and 'unpublished work' should be incorporated in the text.

Artwork should be sent with the manuscript. All artwork should be marked with the author's name. Line drawings should preferably be in Indian ink on heavy cartridge paper, although other materials are acceptable; thin, shiny, folded, torn or heavily handled material should be avoided. Matt rather than glossy photographs are preferred. Figures are usually reduced to one column width. The originals should be about as wide as a page of *Nature*. Figures, particularly maps, should contain nothing but essential material. It is preferred that the original be unlabelled, but with a copy containing lettering. Labelling on photographs should if possible be avoided entirely. We are always glad to see artwork for possible use on the cover, whether or not it is associated with a manuscript, but cannot guarantee the return of material.

In order to save on postal expenses we return only the top copy and artwork of manuscripts that we cannot publish.

A fuller guide appeared in *Nature* (**246**, 238; 1973).

Cover picture

(Cover by Harold Yates.)

Volume 256   **August 21 1975**

Guide to authors

● Articles are up to 3,000 words in length with at least six displayed items (figures and tables) and may either be reports of major research developments in a subject or broader reviews of progress.

● Letters are brief reports of research of unusual and wide interest, not in general longer than 1,000 words; at most they have three or four displayed items (figures and tables).

● 'Matters Arising' permits occasional short discussion of papers that have previously appeared in *Nature*. There is a limit of 300 words.

Manuscripts may be submitted either to London or Washington. Three typed copies should be submitted, each including lettered copies of figures. Typing (including references) should be double spaced. The title should be, brief and informative. Pages should be numbered. References, tables and figure legends should start on separate pages. Experimental detail vital to the paper yet which would interrupt the narrative is best placed in the figure legends. Units should conform to the Système International. Greek characters should be identified in the margin on their first appearance. Equations should occupy single lines if possible, exp (a) is preferred to e^a if 'a' is more than one character. Articles should be accompanied by an abstract of not more than fifty words listing the main conclusions.

References are indicated by super-scripts in the text. See any contemporary *Nature* for style, but note:

(i) To refer to several references by the same author at once, only one reference number need be given.

(ii) Cite first and last pages.

Abbreviations should follow the *World List of Scientific Periodicals*, fourth ed. (Butterworth, 1963-65). Symposia are often difficult to refer to and only published or soon-to-be-published volumes should be mentioned in references. Their publisher and place of publication should be clearly indicated. 'Personal communication' and 'unpublished work' should be incorporated in the text.

Artwork should be sent with the manuscript. All artwork should be marked with the author's name. Line drawings should preferably be in Indian ink on heavy cartridge paper, although other materials are acceptable; thin, shiny, folded, torn or heavily handled material should be avoided. Matt rather than glossy photographs are preferred. Figures are usually reduced to one column width. The originals should be about as wide as a page of *Nature*. Figures, particularly maps, should contain nothing but essential material. It is preferred that the original be unlabelled, but with a copy containing lettering. Labelling on photographs should if possible be avoided entirely. We are always glad to see artwork for possible use on the cover, whether or not it is associated with a manuscript, but cannot guarantee the return of material.

In order to save on postal expenses we return only the top copy and artwork of manuscripts that we cannot publish.

A fuller guide appeared in *Nature* (**246**, 238; 1973).

Cover picture

How does Britain run its science?
See the chart at the centre of this
issue.   (Photo: Michael Freeman.)

Volume 256   August 28, 1975

Guide to authors

● Articles are up to 3,000 words in length with at most six displayed items (figures and tables) and may either be reports of major research developments in a subject or broader reviews of progress.

● Letters are brief reports of research of unusual and wide interest, not in general longer than 1,000 words; at most they have three or four displayed items (figures and tables).

● 'Matters Arising' permits occasional short discussion of papers that have previously appeared in *Nature*. There is a limit of 300 words.

Manuscripts may be submitted either to London or Washington. Three typed copies should be submitted, each including lettered copies of figures. Typing (including references) should be double spaced. The title should be brief and informative. Pages should be numbered. References, tables and figure legends should start on separate pages. Experimental detail vital to the paper yet which would interrupt the narrative is best placed in the figure legends. Units should conform to the Système International. Greek characters should be identified in the margin on their first appearance. Equations should occupy single lines if possible, exp (a) is preferred to e^a if 'a' is more than one character. Articles should be accompanied by an abstract of not more than fifty words listing the main conclusions.

References are indicated by superscripts in the text. See any contemporary *Nature* for style, but note:

(i) To refer to several references by the same author at once, only one reference number need be given.

(ii) Cite first and last pages.

Abbreviations should follow the *World List of Scientific Periodicals*, fourth ed. (Butterworth, 1963-65). Symposia are often difficult to refer to and only published or soon-to-be-published volumes should be mentioned in references. Their publisher and place of publication should be clearly indicated. 'Personal communication' and 'unpublished work' should be incorporated in the text.

Artwork should be sent with the manuscript. All artwork should be marked with the author's name. Line drawings should preferably be in Indian ink on heavy cartridge paper, although other materials are acceptable; thin, shiny, folded, torn or heavily handled material should be avoided. Matt rather than glossy photographs are preferred. Figures are usually reduced to one column width. The originals should be about as wide as a page of *Nature*. Figures, particularly maps, should contain nothing but essential material. It is preferred that the original be unlabelled, but with a copy containing lettering. Labelling on photographs should if possible be avoided entirely. We are always glad to see artwork for possible use on the cover, whether or not it is associated with a manuscript, but cannot guarantee the return of material.

In order to save on postal expenses we return only the top copy and artwork of manuscripts that we cannot publish.

A fuller guide appeared in *Nature* (**246**, 238; 1973).

Staff changes

This week we say goodbye to two members of the *Nature* staff, John Gribbin and Gillian Boucher.

John Gribbin has been with us for five years, during which time he has made his mark, in particular, as a writer, both in *Nature* and for Nature–Times News Service in *The Times*. He leaves to join the Science Policy Research Unit at the University of Sussex.

Gillian Boucher joined *Nature* two years ago, and for the past year has edited News and Views with considerable distinction. She and her husband will be living in Italy for a year or two.

Extra copies of the chart "Who runs British science?" are available from Subscription Department, Macmillan Journals Ltd, Brunel Road, Basingstoke, Hants, UK. Price 30p (including postage).

Ask the young about the future

THE House of Commons Subcommittee's examination of the future of scientific research in British universities seems in imminent danger of falling apart—not through any violent centrifugal tendencies of its members, but because a growing sense of boredom is beginning to surround its missions. This past week, Sir Sam Edwards, Chairman of the Science Research Council, and Sir Fred Stewart, Chairman of the Advisory Board for the Research Councils, two of the men most central to policy-making in science, dourly defended the present system against some tepid questioning, and once more one wondered why we were all there.

Part of the reason for the lack of enthusiasm is that everyone knows that the education and science sector is suffering heavily from the ravages of inflation, and it is thus no time for reasonable men to start bickering among themselves. And the Rothschild reorganisations are too recent in people's memories for there to be much interest in another round of fundamental changes.

So what can the committee profitably do? It could switch its attention to the young students who are potentially a driving force in science in the 1980s. Two questions about them are surely worth extended investigation. First, are they going to continue to desert the physical sciences in large numbers? And, second, what in the educational system leads to the alienation between pure and applied science, which is an undoubted contributor to Britain's poor performance in converting research into development and production? The committee has shown more than a passing interest in this latter issue and might find it a more congenial question, and certainly one with major economic implications. Fiddling with the dual-support system and the structure of the Science Research Council can at most have marginal benefits in comparison, although a detailed look at the alienation problem might well lead, in the longer term, to the fundamental changes which nobody seems keen to contemplate at present.

Is there money still for tea?

IN recent months, the Department of Energy has been stepping up its campaign to persuade the British public to save energy. "It's everybody's baby. The man who drives alone to work. The boiler-minder who could get more out of his boiler. The housewife who lets her kettle boil away while she chats on the phone. Just by being careful, you can save your own money—and millions for Britain." And in another advertisement a kettle is shown with pound notes steaming out its spout.

Our back-of-the-envelope calculations say this. Britons drink some 2×10^8 cups of tea or coffee daily, of which half come from kettles, each kettle producing two cups, say. Of these 5×10^7 kettles, perhaps half have whistles or automatic cut-outs. Further, of the remainder only about 10% are kept boiling for any substantial period—let us say on average one minute. Thus British kettles boil unnecessarily for 2.5×10^6 minutes daily. This wastes roughly 10^5 kilowatt-hours of energy every day. This costs the foolish housewives of Britain £1,500 a day or an average of 0.015p per family.

Hardly pounds streaming out of each kettle, you may say, but worth saving nevertheless. But it costs money and it uses up energy to run a publicity campaign. Advertisers measure success at the fractions of a percent level—a campaign that persuaded 1% of the population to change its ways would

have been remarkably successful. At stake are thus only 10^3 kilowatt-hours of energy or the colossal sum of £15 a day—although the steam heats up the room and thus for at least half of the year might be deemed a good thing.

We haven't dared ask the department how much this particular advertisement has cost but it must be several thousand pounds—and in the process energy has been consumed in printing it, distributing it and raising it on to a placard.

Ironically, really worthwhile rather than trifling savings are possible when boiling a kettle. Few measure the water into the kettle and as a result an enormous amount of water is raised to the boil and then not used. It is left as an exercise for the reader to determine how much the department could have saved—then to devise an appropriate slogan.

Similar arguments can also be applied to domestic hot-water systems. Lagging a hot-water tank is fine, and the Department of Energy rightly points out that considerable savings of money and energy can be made in this way, but how many people heat their domestic hot water for un-necessarily long periods in the summer, and indeed how many people have hot-water tanks that are too big for their requirements?

international news

Exit Dixy, with acrimony

by Colin Norman, Washington

DR Dixy Lee Ray, the controversial and widely-respected former chairman of the Atomic Energy Commission (AEC) last week quit her job as the top science official in the State Department because she had been virtually frozen out of foreign policy decisions, even on matters involving research and development. Her letter of resignation to President Ford, which she subsequently made public, also contained a sharp attack on the Administration's domestic science policies, suggesting that "pitifully little is being done" in several critical areas, including the setting of policy for energy research and development.

Ray's appointment to the State Department job was widely regarded as a sign that the department's science and technology bureau would be elevated to an important position in the departmental hierarchy. But, after five months of being ignored, she handed in her notice on June 20, and aired her grievances in a series of television interviews and in an appearance before a Senate subcommittee. Her complaints paint a stark portrait of bureaucratic warfare in the State Department, and a gloomy picture of the manner in which foreign policy involving science and technology is made.

Appointed chairman of the Atomic Energy Commission by former President Nixon in 1973, Ray's appearance in Washington was greeted with reams of prose concerning her "sensible" clothes, her knee socks, her two dogs which shared her office, and her motorized home in which she lived close by the AEC's headquarters. In a lacklustre Administration, she stood out as a colourful character, but she also quickly established a solid reputation as a forceful administrator of one of the most controversial agencies in the federal government. She forced the resignation of several AEC officials, made the agency much more open and responsive to public concerns, and adopted a tough, no-nonsense approach in her dealings with other agencies and with Congress.

When the AEC was abolished by an

Dixy Lee Ray: gone but not forgotten

Act of Congress which established the Energy Research and Development Administration and the Nuclear Regulatory Commission, Ray accepted the job as head of a new bureau in the State Department, with the cumbersome title of Bureau for Oceans and International Environmental and Scientific Affairs (OES). The Bureau was foisted on the State Department by Congress, in a bill passed in 1973, but which was not put into effect by the department for more than a year—a fact which indicated the importance which Secretary of State Henry Kissinger attached to the matter. The new bureau is an amalgamation of several former offices; it was supposed to inject high-level analysis into policies concerning science and technology—such as export of nuclear materials, the world food shortage, ocean research and so on.

Kissinger is, however, well-known for ignoring the State Department's bureaucratic machinery in formulating foreign policies, and relying instead on advice from a few key associates. Ray was never one of those associates, and she said last week that by the time her bureau became involved, policies were already established. The bureau, for

example, played no role in negotiations concerning the controversial agreement between West Germany and Brazil on the sale of nuclear materials, matters concerned with the Law of the Sea Conference are handled by another office, and the prime focus of energy policymaking in the State Department is in the Office of Economic and Business Affairs, which is headed by Thomas O. Enders, a close associate of Kissinger's.

In her letter to Kissinger, Ray said that "for some time I had hoped that my office and the Bureau I head would play a significant role in the formulation of the Department's science policy . . . unfortunately, that desirable condition has not been fulfilled. Many of the areas for which OES has statutory responsibility are, in fact, being pursued in other bureaux and offices." Ray subsequently told the Senate subcommittee on oceans and international environment, which was chiefly responsible for the bill establishing OES, that she hadn't seen Kissinger once since she was appointed. She had asked for a meeting to discuss her concerns, she said, but was told to prepare a "briefing paper" instead.

In a longer letter to President Ford,

Ray said "the (OES) Bureau can do little but acquiesce in the policies set by others, and attempt to implement its broad responsibilities with little authority and few resources". She added that "similar problems plague our Nation's domestic science policy ... I am deeply concerned that the imperative to use existing and proven technology for vigorous attack on today's problems is not fully recognised nor appreciated at the highest levels of government."

Ray went on to point out "pitifully little is being done" in the area of energy resources and technology. "Our country is drifting", she said, "we seem neither to have the will to conserve energy nor the courage to map out a national program that will free us from the bondage of too great a reliance on imported energy".

After delivering herself of her grievances, Ray departed last Friday to her native Washington State in her motor home, to complete a book aptly titled "Good-bye America". Washington DC will miss her.

● SUMMER has settled in on Washington, bringing with it mind-boggling allegations of wrongdoings by the CIA, utter indecision in Congress over energy policy, growing speculation about next year's presidential elections, and a smog level officially deemed hazardous to health. Against that background, the mixed political fortunes of the National Science Foundation (NSF) haven't been accorded much attention by the daily press, but last week the House of Representatives took a couple of significant votes on NSF's activities.

The House approved an appropriations bill which would slash $44.3 million from the Ford Administration's proposed budget for NSF for the financial year which began on July 1, ensure that basic research would bear the brunt of that cut, and stop the foundation promoting the use of school science courses. The bill now goes to the Senate, where its prospects are uncertain, but whatever the Senate decides, the hostility toward NSF so far demonstrated by the House is cause for some concern.

The prohibition against promoting school science courses is particularly noteworthy. The NSF has for years been developing innovative school science curricula, which it has promoted by sponsoring courses for teachers to acquaint them with the new materials. But this practice ran into right-wing criticism in the House earlier this year over an NSF-sponsored course called MACOS (Man: A Course of Study). That criticism, in fact, led to many of NSF's other political problems.

Briefly, a number of conservatives in the House attacked MACOS because it depicts the rough realities of Eskimo life rather explicitly in a number of films and books. The leading critic, Representative John B. Conlan, a Republican from Arizona, has described the course as undermining traditional American values, and he objected to the fact that NSF was promoting it against other, privately developed, courses. Conlan proposed an amendment to an NSF bill in April to stop NSF promoting school science courses because, he said, such activities are leading government control of the content of local education. His amendment was narrowly defeated, but the furore which ensued led the House Committee on Science and Technology, and the National Science Foundation itself, to conduct a thorough review of NSF's education programmes.
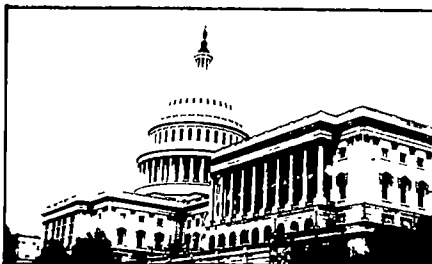
In spite of the fact that Conlan's amendment was defeated, the House Appropriations Committee approved a budget bill for the foundation on June 20 which expressly forbids NSF from spending any money on promoting school science courses. The committee noted that MACOS is now being taught in 1700 schools throughout the United States, and suggested that use of government funding for promoting particular courses gives them an unfair market advantage. The House adopted the committee's recommendation with scarcely any debate.

The irony in all this is that Congress has in the past been critical of NSF for doing too little to implement the courses, research findings and so on, which result from activities it has funded. Moreover, many educators have pointed out that school science education was in a mess before NSF arrived on the scene.

As for other parts of the NSF appropriations bill, the House decided that NSF should have $727 million to spend in the 1976 fiscal year (including $20 million in funds carried over from 1975), which is $44.3 million less than the Administration requested. The House, moreover, suggested that $35 million of the cut should be taken from the budget proposed for research grants. If accepted by the Senate, the bill would provide a total of $345 million for research grant support, which is an increase of only $5 million over last year's level. The House Appropriations Committee, which recommended the reduction, said that this was a

proper level of funding to maintain.

● The second significant vote on NSF taken by the House last week could, however, provide some relief for that beleaguered agency. A House-Senate conference committee was appointed to decide the fate of, among other things, the so-called Bauman amendment to give Congress power to veto individual NSF grants before they are awarded. The amendment had been attached to the House version of the NSF authorisation bill, but it was not approved by the Senate. When the conference committee was appointed, the amendment's sponsor, Robert Bauman, a Republican from Maryland, proposed a resolution instructing House members of the conference committee to ensure that the amendment is written into the final version of the bill. The resolution was defeated by 127 votes to 284, and it is now considered likely that the Conference Committee will water down the amendment.

● The trouble-plagued liquid metal fast breeder reactor (LMFBR) programme, which is the costliest energy research and development effort in the United States, survived a crucial test in Congress last month. A move to delay construction of the proposed LMFBR demonstration plant for 18 months, while the entire programme is evaluated, was defeated in the House of Representatives by a wide margin—136 votes to 227. A similar move is likely to take place in the Senate next week.

The challenge to the breeder programme came during a debate on a budget bill for the Energy Research and Development Administration. An amendment to delete funds for the demonstration plant and to prohibit ERDA from buying capital equipment for it, was proposed by Representative Lawrence Coughlin, a Republican from Pennsylvania, who argued that the programme should be postponed until it is determined whether or not the breeder is needed. The National Academy of Sciences is among the organisations which is looking into the LMFBR programme.

Although the amendment was heavily defeated, opponents of the LMFBR programme are not too despondent. A year ago, they point out, such a move would probably have gathered only a handful of votes, but unease over some aspects of the LMFBR programme, including its projected cost of $10.7 billion, caused nearly a third of the House to vote in-favour of a delay. It is, nevertheless, generally agreed that this is the critical year for the LMFBR programme. If Congress now sanctions large expenditures on the LMFBR demonstration plant, that investment will make it difficult to turn the programme off in later years. □

A CONTEMPORARY odyssey of pain, repression and, finally, of achievement, reached its climax this week at a ceremony on the Mount Scopus campus of Jerusalem's Hebrew University, in which an outstanding 26-year-old mathematician was awarded a PhD and the prestigious Aharon Katzir Prize. It began six years ago in the town square of Riga, Latvia, where that same young man, Ilya Rips, set himself on fire in order to dramatise his opposition to the Red Army invasion of Czechoslovakia and to Soviet anti-Semitism. Fortunately, passers-by snuffed out the flames and rushed him to a hospital.

Rips was subsequently sent to join other dissidents at a mental institution, where he spent over two years "under treatment". Had it not been for wave after wave of international protests, he would probably still be there From the time he arrived here in January 1972, until this week, Rips—a naturally very reticent person—stayed out of the headlines. He was content to learn Hebrew, which he now speaks with great fluency, and then to immerse himself once again in the world of mathematics, where his work on the dimensional sub-group problem, begun in Riga, has already aroused international interest. Now he is to be a lecturer in the Hebrew University's Mathematics Department.

● Dozens of government personalities were at the Hebrew University ceremony, but few of them have any idea what is really going on in the lecture rooms and laboratories of local institutions of higher learning. Alarmed at the possible consequences of this situation, Israel's academic centres have taken it upon themselves "to educate" the country's policy-making elite. At the Weizmann Institute alone, day-long seminars on various aspects of science have recently been held for members of the Knesset (Israel's Parliament) and for senior officials of the Ministries of Finance, Commerce and Industry, Foreign Affairs and Education. Although the participants sometimes seem to be slightly bewildered by talk about polymers, antigens and quarks, they generally manage to retain their perspective. For example, when a Weizmann Institute official greeted a group of Finance Ministry officials, he earnestly explained to them that the seminar was strictly educational in character and was not being held "to sell you the institute".

"Don't worry," a Finance Ministry man replied, "we don't have the money to buy it. But perhaps you'll give us an option on the place."

● Government monetary limitations notwithstanding, official bodies are putting more money into the development of solar energy, which, as Minister of Commerce and Industry Haim Bar-Lev recently stated, they hope will give the country increased 'economic energy'.

Twenty years ago Israel was in the forefront of solar energy research, but interest in the subject rapidly declined here, as elsewhere, when low petroleum prices seemed to obviate any need for what was then a much more expensive form of energy. Nevertheless, Israel is probably at present the world's largest

Letter from Israel
from Nechemia Meyers

user, per capita, of applied solar energy.

Backing up this contention in a paper presented at a recent solar energy conference in Rehovot, Dr Harry Tabor pointed out that:

(1) The Dead Sea Works, with approximately 100 km² of evaporation ponds, use solar heat equivalent to about 30×10^6 tons of fuel oil per year —three times Israel's annual national consumption.

(2) Over 100,000 domestic water heaters (which save about 100,000 tons of fuel oil that would otherwise be required for heating water) represent about 0.1 m² of flat-plate solar collector per capita.

Tabor sees justification for using solar energy more widely to heat water, to heat and cool buildings and even to provide a power source for desalination. He argues that if solar ponds could be built for less than $10 per m² —which seems feasible—it would be cheaper to use solar energy for desalination than fuel costing $80 a ton.

● Whatever may be the value of Israeli solar energy research, local agricultural research proves its economic significance year after year. Just recently, for example, scientists at the Hebrew University's Faculty of Agriculture reported on the development of new techniques which should facilitate overseas sales of two important export crops—citrus and flowers.

Confronted with the fact that oranges grown north or south of Israel's traditional Coastal Plain citrus belt tend to have rough peels, which makes them unsaleable in Europe, Shaul Monselise, Raphael Goren and Yair Erner, of the Hebrew University, found a way 'to smooth the peels' with a synthetic growth retardant.

Peel roughness is caused by excess levels of gibberellic acid and cytokinins, hormones which control cell division and cell enlargement in the oranges (as they do in other plants). Since there is an excess of these growth regulators in the early stages of the fruit's growth, the team found that peel roughness could be controlled by spraying Alar (a growth retardant) on the citrus during that period. This cut down roughness by 30% without affecting the fruit's nutritional value or making it unsafe to eat. Now equal sucess is being achieved with a less expensive chemical (CCC).

● Israeli agricultural research is distinguished not only by its quality, but also by the fact that it reaches the farmers and the fields much more quickly than it would in other countries, even those with well organised extension services. This is partly because one-third of Israel's agricultural students are themselves settlement members, who rush back home with any promising new hybrid or hormone. In addition, local farmers are not content to wait quietly for solutions to their problems; instead, they keep pestering the professors for assistance and, moreover, they are willing to try innovative methods even when they are still experimental.

Agricultural know-how reaches Arab farmers almost as quickly as it reaches the members of Jewish settlements The 17 Arab students at present enrolled at the Hebrew University's Faculty of Agriculture, as well as Arab graduates, bring new methods to their home villages (inside the country's 1967 borders) and to the West Bank and the Gaza Strip. Production has risen spectacularly in these latter areas since Israeli troops, closely followed by Israeli agricultural advisers, entered them eight years ago.

This rapid transfer of new forms of cultivation, new seeds, new fertilisers and new pesticides could pose a problem for Jewish farmers, because it deprives them of the technological advantage which they would otherwise have over Arab farmers. Indeed, certain crops that were once grown mainly by Jewish farmers, such as strawberries, have been taken over almost completely by Arab farmers, who have access to the same agricultural research and, at the same time, spend less on manpower because their own large families provide a bountiful source of practically free labour.

Yet the Dean of the Hebrew University's Agricultural Faculty, Professor Isaac Harpaz, is not unduly worried. He points out that the economies of Israel and the Administered Areas are closely linked, and likely to remain so whatever form of political settlement is eventually achieved Thus prosperity for Arab farmers benefits the entire economy.

THERE is a good deal of confusion at the Pasteur where a lively campaign is being waged, by means of handbills, rumours and articles in the press, against the genetic manipulation experiments which have just started there.

The story begins a year ago; indeed when the Asilomar Conference of February 1975 gave the go-ahead to the genetic manipulation experiments which had been temporarily halted, French biologists who wished to do similar research were hardly taken unawares. As early as November 1974, they had asked first the CNRS and then the Délégation Générale à la Recherche Scientifique et Technique to set up an organisation to exercise some form of control over research which nobody denies could possibly be dangerous. Two committees were formed; one, with the task of ruling on the ethical problems arising from the experiments, was chaired by J. Bernard, who is Director of the Research Institute for Diseases of the Blood in Paris. It is made up of J. Monod, F. Jacob, F. Gros, R. Monier, J. P. Ebel, Y. A. Chabert and P. Slonimsky.

The other is a committee of experts comprising 15 researchers, doctors and biologists. With the final document of the Asilomar conference as a guideline, they defined the safety limits to which the experiments submitted to them must conform. The parent organisation (CNRS, INSERM, etc) would then be responsible for the effective control of the experiments. In this way, for example, a subcommission on hygiene and safety will soon be set up at the Pasteur.

Asilomar and the Pasteur Institute

from the staff of La Recherche

At the same time, the DGRST gave a grant of 300,000F to build a laboratory to house 'the moderate risk' experiments in the Pasteur. It was there that the so called 'low risk' experiments were initiated—those which had not been delayed in order to wait the Asilomar conclusions; and it was there that the 'moderate risk' experiments were to be started which could result in new vaccines.

The internal meetings at the Pasteur were to inform the whole staff, both researchers and technicians, of the results of the Asilomar conference and of the experiments which were to be carried out.

Nobody expected the explosion which followed these meetings, the first of which had taken place in April. One group of research workers and technicians undertook to fight both the decision to place the special laboratory within the department of molecular biology and also the experiments themselves. An atmosphere of panic overran the Pasteur. Institute biologists and others formed themselves into a 'biological-information group' and recently they have published a manifesto demanding the suspension of experiments and a public meeting where the advantages and disadvantages of the experiments could be debated.

The dissenting movement is very large. It has to be seen within the political and philosophical framework which brings into question genetic manipulation as well as other biological research, virology, genetics etc., where the dangers must be weighed against expected benefits. The movement contests the present reliance on the scientific expert. It questions the way in which science is run, both at the Pasteur, and in general. And finally it poses the problem for whom is science done, scientist or people?

A NUMBER of choices and decisions must be taken this month and next to rationalise international effort on energy research and development, which, otherwise, promises to be a farce or to duplicate what is being pursued nationally in several places. Perhaps the most significant of these moves is the one Dr Walter Marshall, Chief Scientist at Britain's Department of Energy (and Director of Harwell) claims as his personal responsibility. This is a procedure available within the 1974 Kissinger-initiated International Energy Agency (IEA) for developing commercially valuable products on a consortium basis and for protecting these by licencing.

Marshall points out that it is easy to collaborate internationally on basic research and on the exchange of results, but very difficult to do work of commercial value where governments are involved, particularly the US government. This is first, because of the difficulty of interlocking government management, industry and government laboratories. International schemes which involve the US federal government, for example, fall foul of the American system whereby all information derived through federal participation must be freely shared with American industry. Some months ago Marshall successfully argued that commercial undertakings should be in-

Marshalling our power

from Angela Croome

cluded in the agency's programme and that industrial protection was needed for non-American industrial participants.

Only 10 days ago a compromise agreement was reached after several midnight sessions and this has been recommended to the British government for endorsement. It provides a means of setting up industrial consortia with defined objectives and the production of industrial 'products' for licencing. Member countries of the IEA can opt out of any of them. When the product has emerged the countries which had not chosen to join in that particular project would have either to buy a licence to manufacture or make a down payment later.

Nine research themes for the IEA have been chosen so far, including coal, energy from municipal and industrial waste, radioactive waste management, fusion use of waste heat, energy conservation, solar energy, hydrogen conversion and nuclear safety. The concept of the 'lead country' is, in the main, being adopted in the organisation of the work, existing investment and effort being the leadership criteria.

Of the programmes already well developed, for example, fusion work is likely to be led from within the EEC but application is still too far off for a commercial consortium to be needed.

Britain may well take the leadership on coal utilisation research and the fluidised bed development has now reached the consortium stage. Britain has also put money into its own radioactive waste programme so would be expected to participate in the agency's.

Many of the IEA themes are duplicated under the EEC's joint programme, but the organisation and decision-making structure of the latter is very different. The requirement that all EEC decisions have to be unanimous results in decisions being political rather than scientifically based. Whatever may the point of view of the advisers of other EEC countries, Marshall expects to get better value out of the IEA. The budget for Europe's Joint Research Centre is still under review, though expected to be settled this month, and there is every likelihood that most of the proposed programme will be adopted and for this there are obligatory contributions. For EEC members such as Britain, therefore, it seems logical for the IEA to subsume many of the same programmes. By routeing participation through the EEC, member countries should avoid paying twice. □

Weather wise

from Vera Rich

THE recent talks in Geneva between Soviet and US delegations on the banning of climatic manipulation for military purposes has focused attention, once again, on the draft convention dealing with weather warfare which the Soviet Union presented to the United Nations last autumn, and which, at the time, was viewed in many quarters as little more than a kind of appendix to a general concern with problems of the environment.

Russia has, of course, a long history of climatic warfare, and her secret allies 'General January' and 'Field-Marshal February' have, through the ages, proved the downfall of Swedes, Poles, French and Germans. In a situation of conventional warfare, the Russian army has only to retreat sufficiently far and sufficiently slowly for the invader to be trapped by winter —and the climate does the rest. But, endowed as she is with a natural strategic weather advantage, the Soviet Union has been quick to see the possibilities of artificial climatic manipulation. Recent articles in *Mezhdunarodmaya Zhizn'* and *Krasnaya Zvezda* have drawn attention to a number of possible artificial meteorological weapons, ranging from the melting of the Arctic ice and tsunami (tidal waves) caused by nuclear explosions on the edge of the continental shelf to the use of infrasound and atmospheric activity to produce psychotropic effects—depression, terror, and the "suppression of mental activity"—in large groups of the enemy in specific target areas. According to the articles in *Mezhdunarodmaya Zhizn'*, the Americans are researching the possibility of "changing the nature of lightning", with a view to being able to direct electrical charges of "tremendous" power against specific targets.

It would be interesting to know the source of this last piece of information. When Academician Kirillin, the Soviet Minister of Power, visited Britain in May 1974, he was shown an impressive experiment at the Culham Atomic Energy Research Establishment in which lightning striking an aircraft in flight was simulated. Could this have been interpreted as a front for some sinister NATO—and therefore, in Soviet eyes, American—weapon?

Some of the other potential weapons detailed in the Soviet press verge on the fantastic—a localised window in the ozone layer to admit ultraviolet radiation, for example—and it seems difficult to see why Mr Brezhnev has found it necessary to stress the urgency of new agreements banning development in this field. Perhaps the urgency is more a matter of politics than immediate military danger. As a preliminary to the projected Ford–Brezhnev summit meeting, it would be gratifying if the USA and the USSR could reach some form of international agreement in the cause of peace and friendship. And what could be easier than for both sides to renounce the use of a weapon that neither actually possesses at the present time. □

THEOLOGY was once known as "the queen of the sciences", and although an examination of recent *Nature* indexes does not produce many references to this subject, I think it may be permissible to mention the idea of the "God of the gaps". I have unfortunately been unable to trace the origin of this phrase, which is used to describe the views of those who are unable to come out clearly as rationalistic atheists. They hang on to the idea of a deity, but restrict his activities to the gaps between the major fields of man's activities, where science is thought to be able to give a full explanation.

My interest in this theological proposition was aroused when I realised that it was very similar to the view held by many leading conservationists. They say that they believe that wildlife and countryside preservation is important, but they acknowledge that the interests of the farmer and of food production must have priority in any scheme for managing the rural landscape. This point is explicit in the recent report of the Countryside Commission, *New Agricultural Landscapes*. Any wish to retain the familiar pattern of hedgerows, picturesque buildings and flower-rich though rather unproductive meadows is castigated as sentimental. We are urged to try to make the best of the inevitable.

It must be admitted that, even within this pattern of farming development, useful compromises which have done much to spare wildlife (particularly birds) have been possible. There have now been many 'Silsoe-type' exercises, for example. These follow the pattern

Don't ditch hedging

KENNETH MELLANBY

of the first weekend conference at Silsoe in Bedfordshire, when agriculturalists and conservationists tried to work out various schemes for managing a farm where nearly maximum productivity could be married with the least damage to the native flora and fauna. Small, unproductive patches on the farm are identified, and these are planted with trees and shrubs to act as mini-nature reserves.

All these developments result, however, in some impoverishment of the landscape and a reduction in numbers of many native plants and animals. This is accepted because of the belief that we must do everything possible to maximise food production, as otherwise we may all face malnutrition at the best and mass starvation at the worst.

A country like Britain that imports nearly half its food cannot enjoy the luxury of 'wasting' any area for conservation if it can be used for food production.

I believe that the time has come for conservationists to be much more aggressive. Farmers have a right to make a reasonably good living from what may be a very difficult and exhausting job. The government has the duty to see that Britain's food supply is safeguarded, even if the pound sterling falls in value so that we cannot continue to import so much of what we eat. There is a good chance that in a few years the growing world population will absorb any surpluses, and that we will be unable to make good food deficiencies by imports. All these points are taken as arguments in favour of maximising productivity and treating conservation with caution.

I do not believe, however, that the choice is really between the risk of starvation with a rich and varied countryside, and enough food with an impoverished landscape. Britain already produces enough food to provide its population with an adequate diet, with enough calories and protein for all. Imports are mainly used to feed livestock to provide meat, which is produced for enjoyment rather than to prevent any malnutrition. The choice is really between two forms of enjoyment —a meat-rich diet or a countryside rich in wildlife. So the conservationist need no longer be content with the gaps—he can come out in to the open and rightly demand his share in shaping the future pattern of all parts of the rural landscape.

news and views

Viruses and histocompatibility antigens: an unexpected interaction

from E. Lennox

IN the minds and activities of immunologists and immunogeneticists those highly polymorphic cell membrane proteins, the major histocompatibility antigens, loom large. These proteins and the products of other genes closely linked to the ones that specify them dominate a wide range of immunological phenomena, among them reactions to tissue transplanted from one individual to another and reactions in culture of mixtures of lymphocytes from different individuals.

In fact thymus-derived cells in particular seem to react strongly and with high frequency to histocompatibility antigens of other members of the species (for example Howard and Wilson, *J. exp. Med.*, **140**, 660; 1974), and even to those of other species (Lindahl and Bach, *Nature*, **254**, 607; 1975). But transplantation and the mixing of lymphocytes in culture are devices of the experimentalist. To what natural phenomena do they correspond? How would the cells of one individual know the histocompatibility antigens of another? Explanations have been sought in phenomena that do lead to cell mixing: fertilisation and gestation (in mammals, at least), sorting during differentiation, transmission of cancer cells (see Bodmer, *Nature*, **237**, 139; 1972; Burnet, *Nature*, **245**, 359; 1973) but none have rung the bell of general acceptance. In fact it can be argued (for example, Cohn, in *Genetic Control of Immune Responsiveness*, edit. by McDevitt and Landy, 427; Academic, New York and London, 1972, and Lafferty and Cunningham, *Aust. J. exp. Biol. med. Sci.*, **53**, 27, 1975) that the apparent importance of the major histocompatibility antigens is exaggerated by the peculiar relation to the immune response and that other polymorphisms are probably equally important.

Now, a new element has been added: in an infected animal, virus-infected cells are vigorously attacked by thymus-derived lymphocytes and the antigen they see is a major histocompatibility antigen modified in a way specific to each virus. Since, moreover, viruses do travel from one individual to another, it is natural to seek an explanation for histocompatibility antigen polymorphism in terms of this remarkable phenomenon (Doherty and Zinkernagel, this issue of *Nature*, page 50).

In their first reported experiments with lymphocytic choriomeningitis (LCM) virus (Zinkernagel and Doherty, *Nature*, **248**, 701; 1974) injected into mice of different histocompatibility types (*H-2* type) they detected cytotoxic T lymphocytes that would kill only LCM-infected cells of the same *H-2* type as the donor of the cytofoxic lymphocytes. This suggested two possible explanations: either cytotoxic lymphocytes, in order to kill, had to match their *H-2* with that of the target or the antigen to which the lymphocytes were sensitised is a virus-induced modification of the histocompatibility antigen of the cell target. Experiments by them and others indicate that the latter is the more likely.

About the same time Blanden (in *Progress in Immunology*, **2**, vol. 4, edit. by Brent and Holborow, 117; Associated Scientific, Amsterdam and New York, 1974; and Gardner, Bowern and Blanden, *Eur. J. Immun.*, **5**, 122; 1975) reported similar findings with ectromelia virus in mice and had shown earlier that the new antigen produced by LCM and by ectromelia in cells of the same histotype were distinct. The total lack of relationship between LCM and ectromelia makes their similar effects on H-2 antigens even more striking. Further experiments have defined more precisely the requirements for *H-2* matching of the virus-infected generator of cytotoxic lymphocytes and of the target (Blanden *et al.*, *Nature*, **254**, 269; 1975).

By using mice of strains with recombinant *H-2* haplotypes on the same genetic background, they were able to show for LCM- and ectromelia-infected mice that *K* or *D* end matching of the generator of the cytotoxic lymphocytes and of the virus-infected target sufficed. This confirmed their previous findings that *M* locus and minor histocompatibility differences were unimportant. Of special interest was the observation that matching of the *I* region (the region of the immune response genes and of the I antigens) was not essential but may exert a minor effect. This tends to emphasise a different kind of mechanism than the *H-2* region matching required for T cell help of B cells for in this case *I* region matching is necessary (Katz *et al.*, *J. exp. Med.*, **141**, 263; 1975).

There have been attempts to elucidate the mechanisms by which viral infection generates a new specificity that depends on the particular virus and the haplotype of the infected cell. Experiments on a seemingly unrelated system provide clues. Shearer (*Eur. J. Immun.*, **4**, 527; 1974) had shown that coupling of the trinitrophenyl group (TNP) to the surface of spleen cells made them good inducers of cytotoxic lymphocytes when cultured with spleen of syngenic mice. Moreover, the major cytotoxicity was directed not at TNP itself (see also Dennert, *Nature*, **255**, 712; 1975), but at the histocompatibility antigen, modified by reaction with TNP. In the virus-infected cell one might imagine an interaction of H-2 protein with viral protein to induce appearance of previously unrevealed determinants in a way that is specific for the H-2 protein and the viral protein.

In favour of this mechanism are several observations. First, the amount of normal H-2 on ectromelia-infected cells seems reduced, for they are less sensitive to killing by lymphocytes specific for their unmodified H-2 (Gardner *et al.*, *Eur. J. Immun.*, **5**, 122; 1975). Second, with vaccinia-infected cells and cytotoxic lymphocytes from vaccinia-infected mice, anti-H-2 serum acting on the target will block cytotoxicity (Koszinowski and Ertl, *Nature*, **255**, 552; 1975). Needless to say, similar H-2 matching of the generators of cytotoxic lymphocytes and of target cells are required as for LCM and ectromelia (Koszinowski and Thomssen, *Eur. J. Immun.*, **5**, 245; 1975). In addition, Koszinowksi and Ertl show that anti-vaccinia serum, not cross reacting with H-2, also blocks. Finally, the amount of H-2, measured by absorption, is reduced on infected cells and preincubation of them with anti-H-2 reduced the amount of anti-vaccinia that could bind. All this

indicates a close physical relationship between H-2 and the viral protein that might induce the H-2 protein to reveal new determinants and hide old ones.

Now we are back full circle. What can all this have to do with histocompatibility antigen polymorphism? Doherty and Zinkernagel (page 50) attempt to tie it to a mechanism for enhanced resistance to viruses. They suggest that the assured heterozygosity at the two ends of the *H-2* locus in a random breeding population gives the virus four different proteins to modify, two at the *K* end and two at the *D* end. This would induce a more vigorous immune response. In support of this they compare the lytic activity of lymphocytes of LCM-infected CBA mice (H-2^x) on cells of:

$$\text{type 1:} \quad \frac{K^x D^x}{K^x D^x};$$

$$\text{type 2:} \quad \frac{K^x D^x}{K^b D^b};$$

$$\text{type 3:} \quad \frac{K^x D^d}{K^b D^d}.$$

Compared to the activity on homozygous *k* cells (type 1), the activity on heterozygous *k/b* (type 2) is reduced as it is on the homozygous recombinant *k/d* (type 3). Moreover F1 mice infected with LCM show stronger immune responses than either parental strain.

It is easy to flaw their proposal either on grounds of plausibility or experimental details. The effects they measure on cytotoxicity are not large and in fact that measured on the heterozygous target and on the homozygous recombinant differ as much from each other as from that on the homozygous parent. In the *in vivo* experiments, the parental strains are not congenic, thus many other factors might be involved in the increased response of the F1. Indeed Koszinowski and Ertl show with vaccinia that F1 mice generate more cytotoxicity when measured on cells of either parent than do the parental strains themselves. In any case, for the argument of Doherty and Zinkernagel, LCM is an unfortunate choice. In this case the augmented immune response in F1 mice leads to quicker death and higher lethality for a given virus dose. This may not be true for all viruses of course (for example Blanden, *Transplantn Rev.*, **19**, 56; 1974) but it does cool the fervour of the embrace of their proposal.

From the point of view of plausibility, theirs seems a weak argument for polymorphism. In the first place, the animal already has several viral proteins to respond to. In addition even with the mechanism of modifying self proteins, there are many other ways to increase the number of surface proteins available for modification without making them polymorphic. Simple repeated duplications would do. It is true these duplications might drift, but there would be no strong demand for new polymorphisms.

Nonetheless, theirs is an attractive idea and an extension of their proposals might bring histocompatibility polymorphism into a central position. Suppose we return to the observations that, for reasons still unknown, T cells respond with high frequency and vigour to other histocompatibility antigens. If we further imagine that interactions with viral protein makes one histocompatibility protein look like some other member of the set, then the apparent preoccupation of T cells with antigens of this set could be put to good use for controlling viral infection. Polymorphism would be advantageous if the special relation to the immune system were maintained for all variants, and if there were repeats in the set of hidden determinants revealed by interaction of the virus with the polymorphic proteins. Otherwise every new histocompatibility antigen variant in interaction with a virus would give determinants outside the set. In sum, this proposal is a specific form of 'molecular mimicry' of histocompatibility antigens by viral proteins (Snell, *Folio biol., Praha*, **14**, 335; 1968).

And if tumour-specific antigens of chemically induced sarcomas were also members of the same set of histocompatibility antigens as recent experiments indicate they might be (Invernizzi and Parmiani, *Nature*, **254**, 713; 1975) then we might be even closer to understanding polymorphic histocompatibility antigens and the apparent preoccupation of the immune system with them.

Morphine and cyclic nucleotides

from L. L. Iversen

UNDERSTANDING the mechanism of action of morphine and other opiates, and the phenomena of tolerance and physical dependence associated with their repeated use, has been for many years one of the major unsolved problems in neuropharmacology. Important progress, however, is now being made in this area. A major breakthrough was the development of biochemical methods for detecting receptors in brain and other tissues, based on the finding that a specific binding of radioactively labelled morphine-like drugs such as etorphine, or morphine antagonists, such as naloxone, could be detected in homogenates of brain (Pert and Snyder, *Science*, **179**, 1011; 1973; Tirenius, *Acta Pharmac. Tox.*, **32**, 317; 1973; Simon *et al., Proc. natn. Acad. Sci. U.S.A.*, **70**, 1947; 1973; see review by Snyder, *Nature*, in the press). Binding experiments of this type have greatly facilitated studies of the precise anatomical distribution of morphine receptors in the brain, and have helped to elucidate the interactions between morphine-like drugs and morphine antagonists and their receptors.

In a search for a simpler model system in which to study opiate effects, a series of tumour cell lines derived from the C1300 neuroblastoma has been screened for the presence of specific opiate binding sites, using labelled naloxone binding. Although specific binding sites could be detected in several cell lines, a hybrid neuroblastoma × glioma line, NG108-15 (also known as 108CC15) was found to have a particularly high density of morphine receptors (Klee and Nirenberg, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3474; 1974).

This cell line has since been used to test the hypothesis suggested by Collier and Roy (*Nature*, **248**, 24; 1974) that morphine acts as an inhibitor of a prostaglandin-stimulated adenylate cyclase mechanism. Collier and Roy showed that morphine and related opiates blocked the increased formation of cyclic AMP caused by addition of prostaglandin E_1 (PGE$_1$) to homogenates of rat brain. This effect seems to be related to the normal pharmacological actions of morphine because it could be blocked by low concentrations of the specific morphine antagonist naloxone, it was mimicked by the pharmacologically active drug levorphanol but not by its inactive stereoisomer dextrorphan, and the rank order of potencies of several morphine-like drugs in the biochemical test system correlated well with their known analgesic potencies (see Roy and Collier, *Life Sciences*, 1975, in the press). Traber, Fischer, Latzin and Hamprecht (*FEBS Lett.*, **49**, 260; 1974 and *Nature*, **253**, 120; 1975) recently reported that similar phenomena could be observed in the hybrid glioma–neuroblastoma cells which possess morphine receptors. In these cells, as in brain homogenates, morphine and related drugs antagonised the increased formation of cyclic AMP evoked by PGE$_1$ and this effect was stereospecific and blocked by naloxone. Sharma, Nirenberg and Klee (*Proc. natn. Acad. Sci U.S.A.*, **72**, 590, 1975) made similar observations, and found that the drug concentrations needed for

inhibition of adenylate cyclase by opiate drugs, with or without prostaglandin stimulation, correlated well with the potencies of the same drugs in displacing labelled naloxone from receptor binding sites in these cells.

The results obtained in the hybrid cell line, however, cast some doubt on the view that an interaction of opiates with a prostaglandin-stimulated mechanism is a crucial component in their actions Sharma et al., for example, found several cultured cell lines that showed increased formation of cyclic AMP in response to PGE₁, but only in those cells that possessed specific binding sites for opiates was this response blocked by morphine. Even in those cells that did respond to morphine the drug also depressed basal adenylate cyclase activity in the absence of added PGE₁, an effect not seen in brain homogenates. Traber et al. (1974), furthermore, showed that the interaction between morphine and PGE₁ was noncompetitive. All these results suggest that morphine may not act at the same site as PGE₁.

In their most recent paper (this issue of Nature, page 57), Gullis, Traber and Hamprecht describe a novel action of opiate drugs in the hybrid glioma–neuroblastoma cells. They found that low concentrations of morphine and levorphanol (but not dextrorphan) stimulated the formation of cyclic GMP, while depressing the concentration of cyclic AMP. These effects were blocked by naloxone, which by itself at low concentrations had no effect on cyclic nucleotide levels. At higher drug concentrations a somewhat complicated picture emerged, since morphine, levorphanol, dextrorphan and naloxone all caused increases in cyclic AMP levels The latter effects seem unlikely to be associated with the specific pharmacological actions of these drugs. The stimulation of cyclic GMP formation by low concentrations of opiates, however, represents a novel mechanism that could possibly explain the actions of these drugs in reducing cyclic AMP levels and antagonising prostaglandin effects on cyclic AMP, since reciprocal interactions between the two cyclic nucleotides have been found in a variety of other biological situations.

Recent findings by Klee, Sharma and Nirenberg (Life Sciences, 1975, in the press) also provide new insight into the processes of tolerance and dependence at a cellular level. They found that on continued exposure of the cultured hybrid cells to morphine the cells adapted by an increased adenylate cyclase activity. The cells thus show 'tolerance' in the sense that their cyclic AMP contents are similar to those found in normal cells, in spite of the continued presence of morphine, and

Crossed lines

from Pamela Hamlyn

SINCE human beings are inclined to choose their own mates and to produce a mere handful of offspring, it is hardly surprising that mapping of their genes by classical methods has not been as successful as that of the much more accommodating fruit fly. The mapping of human genes, that is, locating them on the twenty-four chromosomes, is of particular interest as an approach to the understanding of human diseases which arise from the inheritance of faulty genes. It is therefore very fortunate that other methods exist for gene mapping. Of these, the formation of a hybrid cell between two different cell types is probably the most important.

Recently a group of scientists working at the National Institutes of Health (Bethesda) have described two different hybrid cell lines which they suggest might be useful for the location of globin genes, and in addition possibly provide information on genetic factors involved in the regulation of their transcription (Deisseroth et al., Proc. natn. Acad. Sci. U.S.A., 72, 1102; 1975).

One of the hybrid cell lines was formed by fusing Friend mouse erythroleukaemia cells with human fibroblasts. The Friend cells grow in culture and synthesise very low levels of haemoglobin but can be stimulated by the addition of dimethylsulphoxide (Me₂SO) so that 20–50% of the cells give a positive reaction for haemoglobin as detected by benzidine staining. The hybrid cells retain most of the chromosomes of both parents but do not synthesise haemoglobin even when Me₂SO is added to the culture medium, although it can be inferred that at least the β-globin gene is retained. This behaviour—the 'extinction' of the characteristic protein of a specialised cell, when that cell is fused with a relatively undifferentiated cell—is a well documented characteristic of this type of cross. In general it is not clear at what site extinction takes place although conjecture favours transcription of the gene. In these experiments a 'probe' of complementary (c) DNA, made by transcribing purified g l o b i n mRNA with reverse transcriptase, has shown that globin mRNA is not present in these hybrids, suggesting

that extinction takes place at the level of transcription or mRNA processing.

The other hybrid cell line described was formed by fusing the same line of mouse erythroleukaemia cells with erythroblasts from human bone marrow. Erythroblasts are red blood cell precursors. They contain globin mRNA as detected by a cDNA probe and haemoglobin as detected by benzidine staining. These Friend cell–erythroblast hybrids behave quite differently from the Friend cell–fibroblast cross. They retain only four human chromosomes but continue to synthesise haemoglobin provided Me₂SO is added to the culture medium. However, although both parent cells are haemoglobin producers, only mouse globin mRNA is detected in the hybrid cells, suggesting that only mouse haemoglobin is being made.

The authors have explained how they think these hybrid cell lines might be used for gene mapping. They propose that in the Friend cell–fibroblast cross the extinction of the mouse globin genes is controlled by a regulatory locus on the human chromosomes. If further chromosome losses were to occur then this locus might be lost, the globin genes re-expressed, and the chromosome(s) involved in controlling globin gene expression defined. Re-expression of originally suppressed genes has been observed in other cell hybrids so that their scheme might be feasible.

The human globin structural genes are probably located on the chromosome normally lost when a Friend cell–erythroblast hybrid is formed since they are not expressed in these hybrids. To assign the genes to chromosomes more accurately, hybrids will have to be isolated which contain only a few more chromosomes than normal Friend cell–erythroblast hybrids but which do synthesise human haemoglobin. If this is accomplished it will be interesting to compare the gene locations with those obtained by hybridising labelled globin mRNA to chromosomes in situ, a method already used to obtain tentative assignments of globin structural genes (Price, Conover, and Hirschhorn, Nature, 237, 340; 1972).

they show 'dependence' in the sense that when the opiate is withdrawn from the cultures cyclic AMP levels rise to abnormally high values. Recovery from the 'addicted' state is slow, as it

requires the return of adenylate cyclase activity to normal values. These results support the suggestions made several years ago that opiates may act as enzyme inducers in the process of

addiction (Goldstein and Goldstein, *Biochem. Pharmac.*, **8**, 48; 1963; Shuster, *Nature,* **189**, 314; 1961). They also agree with the suggestion by Collier, Francis, McDonald-Gibson, Roy and Saaed (*Life Sciences,* in the press) that abnormal formation of neuronal cyclic AMP in brain plays a key part in morphine addiction, and that some of the features of this state can be mimicked by administration of cyclic AMP or phosphodiesterase inhibitors in order to elevate brain cyclic AMP levels.

Another finding that may offer a crucial key to the understanding of the biochemical mechanisms underlying opiate drug actions is the discovery that chemical substances with morphine-like properties occur normally in brain. Hughes (*Brain Res.*, **88**, 295; 1975) recently described the isolation and characterisation of a low molecular weight peptide from bovine brain that is able to mimic the actions of morphine in pharmacological test systems. The new substance, called 'enkephalin', may be a neurotransmitter or modulator substance made and secreted normally in those brain regions that are rich in opiate receptors, and work on its further purification and identification is being actively pursued in several laboratories.

Although research in the area of opiate drugs is progressing at a bewilderingly rapid pace, it is clear that very important new facts are emerging in this hitherto enigmatic field. The combination of opiates, prostaglandins, brain peptides and cyclic nucleotides in a single branch of research is guaranteed to ensure that it will remain a glamour area for some time to come.

New support for autoimmune basis of myasthenia gravis

from Angela Vincent

The fifth conference on myasthenia gravis was held in New York on May 30–31.

MYASTHENIA GRAVIS (MG) is a disorder of neuromuscular transmission which is relatively rare but can be extremely disabling. It is characterised by weakness and increased fatiguability of skeletal muscle which can be improved by acetylcholinesterase inhibition. Myasthenia is associated with thymic hyperplasia (75%) and thymoma (15%). Antibodies to skeletal muscle and thymic myoid cells have been demonstrated in about 30% of patients, and thymectomy often induces a remission. These features of the disease and its association with other immune disorders led Simpson

(*Scot. Med. J.,* **5**, 419; 1960) to propose that an autoimmune mechanism might be responsible, but attempts to implicate a serum factor blocking transmission have been largely unsuccessful.

Electrophysiological studies on biopsied intercostal muscle from MG patients by Elmqvist *et al.* (*J. Physiol., Lond.,* **174**, 417; 1964) showed reduced amplitude of miniature endplate potentials (m.e.p.ps) and it was suggested that there might be a reduction in the size of individual acetylcholine quanta released from the presynaptic nerve terminal. The possibility remained, however, that there might be reduced sensitivity of the postsynaptic membrane to released acetylcholine. This seems possible, since the total number of acetylcholine receptors has now been shown to be reduced in endplates from MG patients (Fambrough *et al., Science,* **182**, 293; 1973; Green *et al., Phil. Trans. R. Soc.,* **B270**, 551; 1975). However, there are morphological changes in MG endplates (Engel and Santa, *Ann. New York Acad. Sci.,* **183**, 46; 1971) and there is, as yet, no evidence as to whether this reduction in total numbers of receptors represents a reduction in density of functional receptors per unit area.

The first day of the meeting was concerned with neuromuscular transmission in MG and in a new animal model. In the last few years, many groups have purified the acetylcholine receptor (AChR) from the electroplax of electric eel and *Torpedo*. α-Bungarotoxin (α-Bgt), a snake toxin which blocks neuromuscular transmission irreversibly, has been used to assay the AChR in solution and in skeletal muscle. Antibodies raised in rabbits against electric eel AChR cross react with the animals' own AChRs *in situ* and produce progressive weakness, similar in many respects to that found in MG (Patrick and Lindstrom, *Science* **180**, 871; 1973). Several speakers presented results from rabbits immunised against AChR from eel and *Torpedo*. A few days after the second injection, the animals developed weakness, usually progressing to death. Anticholinesterase treatment temporarily improved their condition. A decremental response to repetitive nerve stimulation could be demonstrated and m.e.p.p. amplitude was reduced. Circulating antibodies against electroplax AChR could be shown by *in vitro* techniques and by their ability to block carbachol-induced depolarisation in the intact electroplax.

J. M. Lindstrom (Salk Institute, La Jolla) described immunisation of rats with a single intradermal dose of eel AChR in adjuvants. About 8 days later they developed an acute episode of paralysis from which most recovered

in a few days. This was associated with severe focal degeneration of the muscle endplate and inflammatory infiltration of the muscles (A. G. Engel, Mayo Clinic, Rochester). V. A. Lennon (Salk Institute) showed that this was probably due to cell-mediated hypersensitivity: it could be abolished by prior thymectomy and could be transferred by T (thymus-derived) lymphocytes into X-irradiated recipients. A second chronic stage was also encountered in these animals. It began at about 30 days and was preceded by a rise in serum antibodies against the AChR. Morphologically the endplates had undergone repair, but the new junctions were simpler than normal and resembled those found in MG. There was evidence of neuromuscular block, reversed by anti-cholinesterase, but these animals did not show such severe weakness as the rabbits. The m.e.p.p. amplitude was reduced, however (E. M. Lambert, Mayo Medical School, Rochester), and the difference in clinical findings between rats and rabbits was thought to be due to the larger number of acetylcholine quanta released per impulse from rat terminals.

Is there a circulating antibody to the AChR in myasthenia gravis?

A. Bender (National Institute of Neurological Disease, Bethesda) showed that sera from patients with MG could inhibit an indirect labelling technique for AChR in cross sections of normal human muscle. S Appel (Duke University Medical Center, Durham, North Carolina) and J. Lindstrom were able to detect binding of immunoglobulins from MG sera to Triton-solubilised receptors from normal human muscle;

but this binding did not appear to involve the acetylcholine recognition site of the receptor. Nevertheless, D. B. Drachman (Johns Hopkins University, Baltimore) had induced electrophysiological evidence of MG in immunosuppressed mice by repeated injections of MG globulins over a period of 10 days.

On the other hand, J Keesey (University of California, Los Angeles) was unable to demonstrate any inhibitory effect of MG serum on α-Bgt binding to mouse diaphragm; E. X. Albuquerque (University of Maryland, Baltimore) had found no reduction in acetylcholine sensitivity in rat or human endplates after treatment with serum from MG patients; and injection of serum globulins or lymphocytes into rats produced no electromyographical evidence of neuromuscular block (T. Namba, Maimonides Medical Center, Brooklyn). Were these negative results due to immunoglobulins being unable to penetrate the intact neuromuscular junction? B. Fulpius (University of Geneva), using α-Bgt-labelled muscles and radioactively-labelled anti-α-Bgt in vivo and in vitro, clearly showed that the antibody was able to penetrate and bind to the motor endplate.

The second day was concerned with genetic, immunological and clinical aspects of MG. Histocompatibility antigens HL-A₂ and HL-A₈ were shown to be more prevalent in MG patients than in the normal population and divided the patients into two main groups: HL-A₂ being more common in older patients with thymomas and circulating antibodies to skeletal muscle, and HL-A₈ being associated with young females with thymic hyperplasia and no circulating antibodies (M. J. Oosterhuis, Wilhelmina Gasthuis, Amsterdam). The relative proportion and absolute numbers of T and B lymphocytes in MG were reported by several speakers, but no consistent pattern emerged from most of these studies G. Birnbaum, however (Cornell University Medical College, New York), found decreased T-cell function in young, hyperplastic patients, and a significant rise in T cells and T-cell function after thymectomy Further immunological studies may well be easier to relate to the disease process if tissue typing is included.

The effect of immunosuppressant therapy was discussed and various regimens for steroid treatment suggested. One interesting aspect presented by G. Matell (Karolinska Institute, Stockholm) was drainage of the thoracic duct undertaken in severe cases These patients improved remarkably after 24–48 hours of drainage, although their condition deteriorated within 1–2 weeks of stopping the treat-

ment. In contrast, injection of the cell-free lymph back into the same patients produced deterioration within 30–60 minutes.

The animal models presented at this meeting showed considerable similarity to MG. Circulating antibodies raised against solubilised AChR can bind to, and directly block the receptor in situ; cell-mediated hypersensitivity to the receptor can produce symptoms of an acute nature, and it may be a necessary prelude to the humoral response in rats. Whether there is a circulating antibody in MG directed against the acetylcholine recognition site of the AChR, and capable of directly blocking the receptor, is still doubtful. It is possible, however, that the immunoglobulins that bind to solubilised human receptors, as shown independently by Appel and Lindstrom, have an indirect inhibitory effect on neuromuscular transmission in situ.

Denervation and re-innervation

from Shin-Ho Chung

BEFORE a foetal muscle is innervated, the fibre is 'supersensitive' to the chemical transmitter acetylcholine over its entire length. When a motor nerve forms an excitable junction with the muscle fibre, the chemosensitive area shrinks progressively and becomes restricted to the junction and its immediate surroundings (Diamond and Miledi, J. Physiol., Lond., 162, 393; 1962). A similar process occurs in adult muscle fibres following regeneration after destruction of the nerve (Miledi, J. Physiol, Lond., 151, 1, 1960; Axelsson and Thesleff, J. Physiol., Lond., 147, 178, 1959). Interest in this system stems partly from the fact that, during their supersensitive period, muscle fibres accept innervation from foreign

Pollution from plants

from Peter D. Moore

IT is both well known and a source of considerable concern that the particulate matter of the atmosphere contains a burden of toxic heavy metals (see Nature, 247, 510; 1974). In south-west England attempts have been made to monitor the fallout of heavy metals by analysing their levels in superficial leaf washings and in cuticular layers on vegetation (see, for example, Little, Environ. Pollut., 5, 173; 1973) The reverse situation is described by Beauford, Barber and Barringer in this issue of Nature (page 35); they have shown that heavy metals from the soil are absorbed by plant roots, are translocated and eventually released into the atmosphere in particulate form, perhaps with fragments of cuticular waxes. It has been realised for several years that plants can generate atmospheric particulate matter, but this is the first clear demonstration, using radioactive tracer techniques, that the movement of heavy metals from soil to atmosphere may accompany this process

This being so, it may be necessary to reconsider the use of vegetation as a convenient filter by which heavy metal fallout from the atmosphere can be monitored. It is not usually known what proportion of cuticular and epicuticular metal is derived from the soil through the plant In many situations the soil levels of heavy metals also reflect atmospheric fallout, in which case the leaf washings will still provide a useful index, but this is not so

where metals in soils are derived from other sources.

But perhaps the most important implication of this work, as the authors point out, is that industrial activity is not the sole source of atmospheric heavy metals. The question which must now be asked concerns the respective importance of these two sources. Here an answer may be found in palaeoecological work by Lee and Tallis (Nature, 245, 216; 1973), who analysed the lead contents of peat samples taken from depths down to 2 m at an upland site in Derbyshire. Lead contents of almost 600 p.p.m. were found in the upper 20 cm of peat, but these fall to about 40 p.p.m. at depths below 20 cm (dated by radiocarbon determination at pre 1460 AD). At depths below 125 cm, the leaf level falls yet further to a fairly steady value of about 10 p.p.m These samples predate the Roman conquest, during which there was some lead mining and smelting, hence it must reflect the background level of atmospheric lead, to which the vegetation is presumably making its contribution. Thus, even if this background were entirely due to the production of metal-containing particulate matter from plants, the current industrial and domestic production of lead still exceeds it by sixty times. In the midlands of England, therefore, atmospheric lead production by vegetation must be regarded a very minor contribution to the atmospheric content of heavy metals when compared with human sources.

nerves, with which they normally do not connect (Fex *et al., J. Physiol., Lond.,* **184**, 872; 1966). These phenomena pose several salient questions. For example, what causes the spread of chemoreceptors after denervation? Is the development of high sensitivity to a transmitter substance a prerequisite for the establishment of a synaptic connection? What happens to 'incorrect' junctions when 'correct' nerves re-innervate muscle fibres? An article by Frank, Jansen, Lømo and Westgaard (*J Physiol., Lond.,* **247**, 725–748; 1975) highlights these questions.

It was originally believed that a 'neural factor' released from nerve terminals prevented the spread of receptors beyond the synaptic region. Subsequent experiments showed this to be implausible. When nerve impulses flowing towards the muscle are blocked with an anaesthetic, while leaving the nerve otherwise undamaged, the muscle membrane becomes supersensitive, as it would if the nerve were cut. The development of supersensitivity can be prevented either by chronic nerve stimulation distal to the region of the block, or direct stimulation of the denervated muscle (Lømo and Rosenthal, *J. Physiol., Lond.,* **221**, 493, 1972; Frank *et al.*). These studies show that contractile activity of muscle fibres, innervated or denervated, inhibits the formation of extra-junctional receptors.

Because connections are formed only when muscle fibres are supersensitive to acetylcholine, it is tempting to postulate that the receptors are the membrane component w h i c h directly controls synapse formation. A pointer in this direction is the fact that the formation of contacts between denervated muscle fibres and foreign nerves can be arrested by maintaining the muscle activity by direct stimulation (Jansen *et al., Science,* **181**, 559, 1973; Frank *et al*). However, a recent demonstration (Van Essen and Jansen, *Acta physiol. scand.,* **91**, 557, 1974) that re-innervation proceeds normally in the presence of α-bungarotoxin, which binds irreversibly to acetylcholine receptors, makes such a generalisation premature.

There is conflicting evidence on the fate of 'incorrect' junctions when 'correct' junctions are re-formed. An earlier preliminary report (Cass *et al., Nature,* **243**, 201, 1973; *Nature,* **243**, 185; 1973) claimed that 'correct' fibres render ineffective 'incorrect' synapses in axolotl muscles, while leaving them structurally intact. Such a suppression of 'incorrect' junctions is known to occur in mammalian skeletal muscles during development (Redfern, *J. Physiol., Lond.,* **209**, 701; 1970; Bagust *et al., J. Physiol., Lond.,* **229**, 241; 1973). Frank *et al.* now show that both

foreign and original synapses can co-exist functionally for prolonged periods of time in adult mammalian muscles. Thus the ability to suppress inappropriate synapses is apparently lost in mature skeletal muscle.

The underlying cellular mechanism for afferent–efferent interaction, as illustrated by the denervated neuromuscular system, remains one of the mysteries in neurobiology. During the process of synaptogenesis in the nervous system, a target cell becomes receptive, selects an appropriate input, and suppresses the inappropriate ones. It is only through a combined immunological, biochemical and neurophysiological study of a simple system, such as the innervation of developing or denervated muscle, that we will gain insight into the rules governing the formation of specific nerve connections.

Hole transport in silica

from Andrew Holmes-Siedle

SILICA (SiO₂) is a very important material in solid state physics, electronics and optics. Although the crystal structure of quartz is complex and several inconvenient phase transitions of the lattice occur, the material is, on the whole, very well characterised. The amorphous form, fused silicon, presents a useful example of the simplest form of disorder, the only deviation from order being some variation in the Si–O–Si bond angles. In optics, the near-zero expansion and high ultraviolet transmission are extremely useful and, in electronics, the crystalline form is used in piezoelectric devices and the amorphous thin-film form is an essential part of all current forms of metal-oxide-semiconductor (MOS) device. Of course, the fact that quartz and silicates are the commonest minerals also help to make silica a technologically and economically important material.

The band-gap of SiO₂ is of the order of 9 eV and thus the electronic transport properties are not easily studied. But, as I discussed previously (*Nature,* **246**, 190; 1973), studies of the motion of electrons and holes in this material are now coming on apace. One of the motives for this work is the need to understand carrier transport in MOS systems and another is the general need to operate insulators under extremes of electric field or photon excitation. In the earlier article, the work of Hughes on electron transport in quartz and fused silica slabs—measuring photoconductivity after nanosecond pulses of X rays—was described. At that time, Hughes's results confirmed the general feeling that all cur-

rent was carried by electrons and that holes were virtually immobile, possibly being lattice-trapped in non-bonding oxygen orbitals (see, for example, Di Stefano and Eastman, *Phys. Rev. Lett.,* **27**, 1560; 1971). Some further measurements by Hughes (*Appl. Phys Lett*, **26**, 436; 1975) have now demonstrated a small contribution of the holes to photocurrents. The fact of the smallness is of great interest and will help towards clearing up controversy in the important field of carrier trapping in some insulators.

Hughes's measurements are summarised in the figure, which shows that, whereas electrons travel rather easily through the SiO₂ lattice, suffering only longitudinal optical phonon scattering, holes can essentially be 'frozen' at low temperature but still possess a measurable mobility at room temperature. This explains a hitherto puzzling result of Powell and Derbenwick (*IEEE Trans. nucl. Sci.,* **NS18**, no. 6, 99, 1971) who irradiated a 200 mm SiO₂ film with 10.2 eV light. Although the light was largely absorbed within a few nanometres of the surface, a positive charge sheet was found at the unilluminated side of the film, suggesting a finite mobility of holes or ions at room temperature. Arguments that ions were carrying charge are now unnecessary and, in general, the important work of distinguishing ionic from electron–hole transport effects in thin films, especially in MOS devices, can perhaps now proceed in a more orderly fashion.

The use of pulsed X-ray-induced photoconduction may become a useful general solid-state tool and will clearly
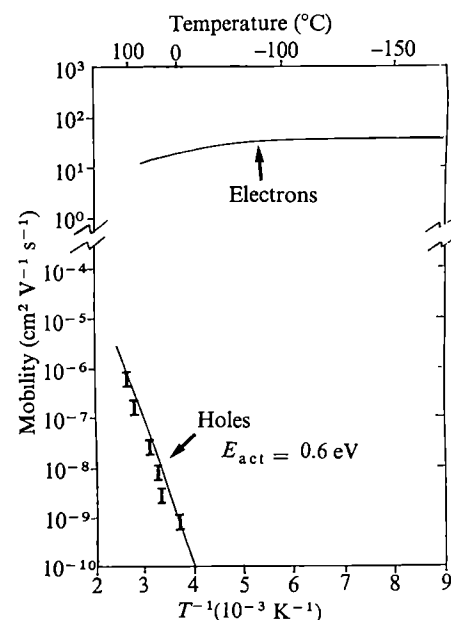
Mobility of holes and electrons in amorphous silicon dioxide films against reciprocal temperature, demonstrating differences in transport mechanism.

play a part in the important general question of charge motion and storage in wide-band solids.

Planetary collisions

from David W. Hughes

AT the dawn of the Solar System collisions between planets could have been so frequent and devastating that we now see only the scattered remnants of this scrummage. Interaction between planets in their present orbits is minimal, a situation which slowly built up as the Solar System aged. But small bodies, such as comets and asteroids, often have orbits which cross those of the planets and thus are intrinsically unstable. They may be eliminated by collision with a planet—either accreting to the planetary surface or (more rarely) being fragmented when they graze the planet's upper atmosphere—or they may be perturbed—either into shorter period orbits with aphelia near the planet's orbit (like the Jovian family of comets) or into hyperbolic orbits which take them out of the Solar System (like the space probes Pioneer 10 and 11).

Probabilities of encounter were first calculated in a classic series of papers by Öpik. Two fundamental approaches can be made to the problem, the analytical one which considers individual particles and their orbital elements and the numerical integration approach which uses random walk and Monte Carlo techniques applied to a large number of particles. These two approaches have been combined by Weidenschilling in a recent paper in *The Astronomical Journal* (**80**, 145; 1975). The probability of encounter between two objects with intersecting orbits is found to be simply a function of their relative velocities and one angular variable. These encounters will eventually occur even between particles with crossing orbits (orbits with the same range of heliocentric distances) because secular perturbations cause nodal precession and advancement of the perihelion, which finally results in intersections and then encounters.

Öpik (*Adv. Astron. Astrophys.*, **2**, 219; 1963) found that the mean lifetime of the Apollo group of asteroids (which have orbits crossing those of Earth and Mars) is 10^8 years. Collisions would have caused their numbers to decay exponentially with time. So if these asteroids are remnants of an original group formed 4,500 Myr ago at the birth of the Solar System, there would have been 2.5×10^{19} of them at $t=0$, equivalent to a mass one hundred times that of the Sun. Clearly this is most unlikely, and thus the asteroids in this group cannot be permanent

members of the Solar System but must be continually replenished from the asteroidal belts and comets.

Weidenschilling comments on the differences in the encounter probability curves of the terrestrial and Jovian planets. This is partly explained by the great mass of Jupiter making it more likely than the inner planets to encounter material. But the mass of the planet also affects the ultimate fate of the small bodies encountered. The terrestrial planets, with orbital velocities several times greater than their escape velocities, have effective cross sections such that the ejection probability is only an order of magnitude higher than the collision probability. For Jupiter the orbital velocity is less than the escape velocity, the collision cross section varies with Joviocentric velocity and about 1,000 more particles will be ejected from the Solar System by Jupiter than collide with it.

Weidenschilling's figure for the probability of ejection by Jupiter is an order of magnitude greater than Öpik's whereas his results for the terrestrial planets agree with those of Öpik. If Weidenschilling's Jupiter result is correct, the time scale for the dynamical elimination of comets must be two to four orders of magnitude greater than the time scale for disintegration. Thus the fact that thousands of dead cometary nuclei are not observed indicates that most comets disintegrate completely or that the dead nuclei are too small to be seen.

The equations given in Weidenschilling's paper also enable the efficiency of cometary ejection processes to be calculated. The author promises to apply these to the production of the Öort cloud of distant comets in a later paper. He also speculates that the total mass and angular momentum lost from the Solar System by cometary ejection might be of cosmogonical significance, indicating that the Solar System's angular momentum–mass balance might have varied with time. □

Type B hepatitis a kissing disease?

from Arie J. Zuckerman

THOUGH the importance of the parenteral and inapparent parenteral route of transmission of hepatitis B infection has long been recognised, it has become difficult to incriminate parenteral transmission in all cases of infection with hepatitis B virus (*Tech. Rep. Ser.* No. 570; World Health Organisation, 1975). Interest in various mechanisms of spread of this infection has flourished in recent years following the experimental demonstration of infectivity of serum by the oral route and epidemiological studies implying the possibility of venereal transmission of hepatitis B (*Nature*, **246**, 59; 1973). Although the reported detection of hepatitis B surface antigen in semen and menstrual blood obtained from individuals with antigenaemia offers possible explanations for infection by the sexual route, the matter is far from settled.

The presence of hepatitis B surface antigen in a variety of body fluids and excretions such as urine, colostrum, milk, saliva, bile, faeces and sweat has not been consistently confirmed. In particular confirmation of the finding of the antigen in bile and faeces is still lacking in spite of intensive studies in various laboratories.

A recent report from Costa Rica has shed some light on possible non-parenteral routes of transmission of hepatitis B in endemic settings (Villarejos *et al.*, *New Engl. J. Med.*,

291, 1375; 1974). Serial samples of faeces, urine and saliva collected from chronic carriers of hepatitis B surface antigen and from patients with acute hepatitis B were tested by radioimmunoassay. The antigen was not found in any of 120 faecal extracts examined. The antigen was detected in three of 130 urine samples and all the positive samples contained occult blood. 274 saliva specimens from 93 chronic carriers were tested and the antigen was found in nearly 60% of specimens belonging to 75 subjects. It is interesting to note that the antigen was not found constantly in the saliva of the carriers, although all had circulating antigen at the time of sampling. Antigen was found in the saliva of 76% of 41 patients with acute hepatitis B, mainly during the first three weeks after the onset of clinical symptoms. The presence of the surface antigen in saliva both in carriers and in patients with the acute disease was not related to contamination with blood. These results show that salivary transmission may be a major mechanism of spread of infection in an area where hepatitis B occurs endemically. Villarejos and colleagues conclude therefore that transmission may occur directly by mouth-to-mouth kissing and in children by exchange of chewed toys and sweets. This presupposes of course that the antigen found in saliva is actually infective.

Prostaglandins and their intermediates

from a Correspondent

> An international conference on prostaglandins was held in Florence on May 26–30.

ARGUABLY the most exciting findings of the whole meeting were revealed by S. Samuelsson (Karolinska Institute, Stockholm) in an introductory address. Recently Samuelsson's group found (Hamberg and Samuelsson, *Biochem. Biophys. Res. Commun.*, **61**, 942; 1974 and *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3400; 1974) that PGG₂ and PGH₂, the endoperoxide precursors of prostaglandins (see *Nature*, **253**, 88; 1975), can, in lungs and platelets, also be transformed into other (non-prostaglandin) products including a hemiacetal derivative 'PHD'. Now Samuelsson's group have identified a very labile intermediate in the conversion of PGG₂ to PHD. This new intermediate does not have a classical prostaglandin structure and has been named 'Thromboxane' (see figure), because it is a very potent platelet aggregating agent. Since it is the first member of a new series of compounds and contains two double bonds it was further designated 'Thromboxane A₂': its metabolite PHD now becomes 'Thromboxane B₂'.

Thromboxane A₂ has at least two other interesting properties; its half-life in aqueous solutions is about 30 s, and it strongly contracts strips of rabbit aorta. As a product of the prostaglandin biosynthesis cascade its generation is of course blocked by aspirin. These properties make it very likely that Thromboxane A₂ is in fact 'RCS'—the mysterious rabbit aorta contracting substance (released from lungs during anaphylaxis), first described by Piper and Vane in 1969 (*Nature*, **233**, 29). The importance of the endoperoxide system in platelet aggregation was emphasised by the Karolinska group's discovery of a subject deficient in the cyclo-oxygenase system (which converts arachidonic acid to PGG₂). Platelets from this subject showed reduced sensitivity to the aggregating action of adrenaline and collagen, but were aggregated by PGG₂. The idea that the endoperoxides themselves, or their non-prostaglandin end products, may be important in other systems was later reinforced by M. Hamberg *et al.* (Karolinska Institute) who found elevated levels of endoperoxide and Thromboxane metabolites in perfused guinea-pig lung during anaphylaxis; PGG₂ and H₂ also had much more marked bronchoconstrictor activity than PGF₂α. Both pieces of evidence suggested that the endoperoxides (or

their non-prostaglandin metabolites) could be important in the anaphylactic reaction. J. Vane (Wellcome Laboratories) presented evidence that PGG₂ and PGH₂ potentiated the inflammation produced in the rat paw by stimuli such as Carraggeenin.

The question of the cellular origin of the substrate required for prostaglandin biosynthesis received some attention, though not perhaps as much as it deserved. Phosphatidylinositol was definitely implicated as a source of arachidonate for, prostaglandin biosynthesis in the pig thyroid undergoing TSH stimulation (B. Haye, S. Champion and C. Jaquemin; Reims-Cédex, France) and in platelets exposed to a variety of aggregating agents (Iacona and Schoene, US Dept. of Agriculture, Beltsville). Some data from Vane's group suggested that phosphatidylcholine was the source of the arachidonic acid liberated during trauma of spleen tissue, and demonstrated that inhibition of phospholipase A₂ could prevent this initial step in the generation of prostaglandins from occurring.

The section dealing with metabolism of prostaglandins aroused less interest: prostaglandin 15-hydroxy-dehydrogenase (PGDH) has been successfully extracted and purified from a variety of sources allowing good kinetic data to be obtained. One particularly ingenious and successful method of purifying the enzyme—by affinity chromatography using prostaglandins

Alternative pathways of arachidonic acid metabolism in platelets. I, Conversion by a lipoxygenase of arachidonic acid to an ω-9 hydroperoxide which is subsequently reduced to the corresponding hydroxy acid. II, Conversion of arachidonic acid by a cyclo-oxygenase to PGG₂. III, Conversion of PGG₂ to a 17C-(ω-6) hydroxy acid and malondialdehyde. IV, Conversion of PGG₂ to PG E₂/F₂ ₐ. This pathway is of minor importance in platelets. V, Conversion of PGG₂ to Thromboxane A₂ and Thromboxane B₂ (PHD). PHD is a hemiacetal derivative of 8(1-hydroxy-3-oxopropyl)-9, 12L-dihydroxy-5, 10-heptadecadienoic acid.

as the ligand—was presented by E. Oliw, I. Lunden and E. Änggard (Karolinska Institute). Throughout the conference, however, many workers were disappointed that so little attention was paid to the question of inhibiting this important enzyme: a really effective inhibitory drug would complement the use of the aspirin-like agents (inhibitors of prostaglandin biosynthesis) as a pharmacological tool to investigate the function of prostaglandins in the body. R. Flower *et al.* (Wellcome Laboratories) presented evidence that PGDH was a short-lived enzyme within the cell, the tissue levels falling very quickly after the administration of protein synthesis inhibitors.

Details of two important radioimmunoassay systems were presented at the meeting: F. Dray, B. Charbonnel and J. Maclouf (Pasteur Institute, Paris) have devised techniques which eliminate most of the artefacts associated with the measurement of E and F-type prostaglandins in plasma and have thus reduced the falsely high readings obtained by many early workers. Their plasma levels for E-type prostaglandins of <5 pg ml⁻¹ and for the F-type of $\leqslant 12$ pg ml⁻¹ are probably the lowest recorded. Because prostaglandins are metabolised so rapidly, it is often more desirable to measure the major plasma (13, 14 dihydro-15 keto) or urinary (tetranor dioic acid) metabolites of prostaglandins. E. Granström (Karolinska Institute) presented some beautiful data on immunoassay systems designed for these purposes. The levels of metabolite were found to fluctuate throughout the oestrous cycle and were depressed during treatment with aspirin-like drugs. Confidence in the validity of both these assay systems was increased by the demonstration that the blood levels found corresponded well with those predicted from daily prostaglandin production rates.

Two clinical syndromes associated with the prostaglandin system were described: the platelet cyclo-oxygenase deficiency reported by Samuelsson's group (see above), and 'hyperprostaglandinaemia'—a syndrome probably due to a deficiency of PGDH—reported by A. Labrum, M. Lipkin and F. Dray (University of Rochester, New York and the Pasteur Institute, Paris). This condition was associated with periodic headache, vomiting, abdominal pain, diarrhoea and pyrexia.

Because of the arrangement of the conference, it was not possible to attend every session, so only certain aspects of the meeting are reported here. But it was clear that the impression felt by many at the end of this exciting conference was that the physiological role of prostaglandins—and now of their intermediates too—was as elusive as ever.

articles

Buried Mesozoic volcanic–plutonic fronts of the north-western Pacific island arcs and their tectonic implications

Jiro Segawa
Ocean Research Institute, University of Tokyo, Tokyo, Japan

Shoichi Oshima
Hydrographic Office, Maritime Safety Agency, Tokyo, Japan

Detailed magnetic surveys around the islands of Japan have revealed the presence of Mesozoic volcanic and plutonic belts at depth beneath the continental shelf. These belts, which can be correlated with those on the mainland of eastern Asia, provide a record of the migration of island arcs since the opening of marginal seas during the Tertiary.

DETAILED surveys (refs 1, 2 and Geographical Survey Institute, Japan, unpublished) have revealed remarkable features in the total magnetic intensity anomalies around Japan and the surrounding continental shelf and magnetic anomalies from Russian data[3,4], and oceanic magnetic lineations compiled by Uyeda *et al.*[5] have also provided evidence of some interesting features (Fig. 1).

Most significant is the discovery of a north–south, positive anomaly belt coupled with a narrower negative belt to the west, running from the eastern side of north-eastern Honshu to the southern part of Hokkaido (Fig. 1 i) with a maximum amplitude exceeding 500 nT. A similar positive anomaly belt runs from the south-east of Hokkaido to Kamchatka, along the Kuril Island Arc (Fig. 1 ii). These two magnetic lineations can be distinguished from lineations in the oceanic basins because their locations coincide with the continental crust. Furthermore, the trend intersects that of the oceanic lineations (Fig. 1 iv) in the western Pacific Basin up to the Japan Trench. Oceanic anomaly iv (Fig. 1) disappears in the middle of the continental shelf in the Japan Trench approximately at the location of the 3,000-m isobath. As the crust under the landward slope of the Japan Trench is not oceanic, the presence there of oceanic anomalies suggests that oceanic crust has been thrust beneath the island arc. By the same reasoning we believe that the positive magnetic lineation (Fig. 1 iii) which is observed at the landward slope of the Kuril Trench and which is roughly parallel to both the trench and the oceanic lineations, is of oceanic origin. Other positive magnetic lineations at ocean-to-continent boundaries occur at Primorye, Sikhote-Alin, Sakhalin and in the Okhotsk Sea (Fig. 1).

The correlations between the positions of the isolated, patch-shaped, positive (or negative) magnetic anomalies in the middle of Honshu and Hokkaido and the line of Quaternary volcanoes known as the Quaternary Volcanic Front (QVF)[6] (Fig. 1) are remarkable. Approximately 30 km east of the QVF (Fig. 1) a Miocene volcanic front has been identified geologically but is not clearly reflected by the magnetic anomalies.

Identification of buried Mesozoic volcanic–plutonic belts

Figure 2 shows profiles of total magnetic intensity anomalies, and bathymetry and magnetisation models which can account for the magnetic anomalies that cross the two continental magnetic lineations (Fig. 1i and ii). The magnetic anomalies along line *A* in Fig. 1 can be modelled by a two-dimensional magnetised block trending north–south, with an intensity of magnetisation 5×10^{-3} e.m.u. cm^{-3}, a direction of magnetisation of N45°W and an inclination of 53°. The magnetised block is 20 km thick with its top 10 km below the ocean. The most important characteristic of this model is that its magnetisation is largely westerly, in contrast to the present magnetic field, which suggests lineation i (Fig. 1) is caused by a westerly remanent magnetisation. Even if the magnetic anomalies are affected by magnetisation induced by the present magnetic field, the westerly tendency of the remanent magnetisation is by no means altered; on the contrary, it would be further emphasised. The magnetic anomalies along line *B* (Fig. 1), on the other hand, can be explained by a two-dimensional block trending ENE to WSW, magnetised with an intensity of 5×10^{-3} e.m.u cm^{-3} in the direction of the present field. Almost the same results can be expected from the other sections of the continental magnetic lineations i and ii.

The fact that lineation i may result from a westerly remanent magnetisation is consistent with the natural remanent magnetisation of the Lower Cretaceous granitic samples obtained by Kawai *et al.*[7] from the Kitakami and the Abukuma massifs in north-eastern Honshu. They proposed an anticlockwise rotation of north-eastern Honshu and a clockwise rotation of south-western Honshu some time after the Lower Cretaceous. As the continental magnetic lineations exist in areas of continental crust, and as a rotation of Japan should accompany any rotation of continental crust, the estimated westerly remanent magnetisation could be further evidence of the anticlockwise rotation of north-eastern Honshu. The magnetisation of lineation ii, on the other hand, cannot necessarily be taken to indicate the absence of rotation with respect to the Hokkaido–Kuril–Kamchatka Arc, because of the nearly east–west geographical trend of the supposed magnetised block. Although it is uncertain whether or not the magnetisation of lineation ii deviated in direction from the present magnetic field, we assume that lineation ii results mainly from a remanent magnetisation with approximately the same direction as the present magnetic field, and that little or
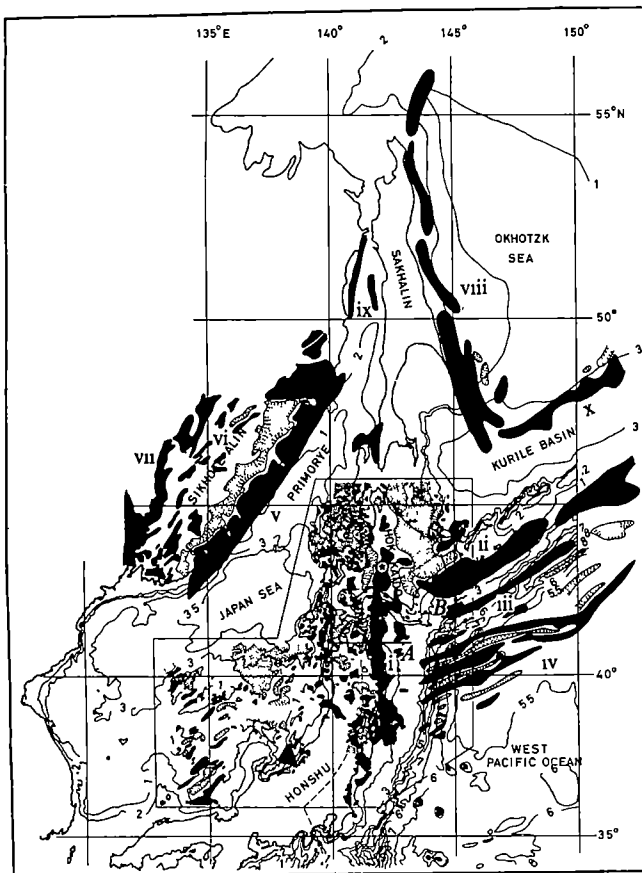
Fig. 1 Total magnetic intensity anomalies in and near Japan. The area enclosed by solid lines has been measured in detail in runs approximately 2 miles apart. Solid areas, positive anomalies; dotted areas, negative anomalies below −200 nT; blank areas, either anomalies between 0 and −200 nT, or no measurements. i-x, Marked lineated anomaly patterns; A and B, locations with magnetic profiles illustrated in Fig. 2; dashed curve, Quaternary Volcanic Front. Bathymetric contours are in metric units. Asterisk, site of a test drilling in the Sorachi region, Hokkaido.

no appreciable rotation has occurred with respect to the Hokkaido–Kuril Arc.

There are two lines of evidence from which the material which causes the continental magnetic lineations can be identified: a test drilling[8] at Sorachi Province, Hokkaido, which coincides with the northern end of lineation i; and the result of measurements[9] of magnetic susceptibilities and remanences of the drilling cores (Fig. 3). The drilling went down to a depth of 3,712.97 m, where the drilling bits hit a basement layer. The basal layer dips from west to east, and the

sedimentary layers show significant unconformities (Fig. 3). Analyses of gravity anomalies combined with geology and drilling results[10] indicate that the basal layer consists of metamorphosed basic volcanics of Lower Cretaceous age, corresponding to the eastern slope of an anticline which aligns with lineation i. The magnetic susceptibilities of the drilling cores (Fig. 3) indicate that the metamorphosed basic volcanics of the basal layer are as strongly magnetised as the recent volcanics. These measurements have also shown that the magnetic Q ratio of the basement rocks is 1 or somewhat smaller. Although it is not certain whether the anticlinal basement is the origin of lineation i, we think it probable that either the anticlinal basement or a belt of ultramafic dyke material beneath it may be responsible for the anomaly. If that is so it can be inferred that volcanic–plutonic activity was initiated in Lower Cretaceous times or earlier, below the lineation. This volcanic–plutonic belt may be compared to the belts of Mesozoic volcanic–plutonic outcrops[11] on the Japan Sea side of south-western Honshu. As for lineation ii, no drilling data are available at present. But the areas are known to be abundant in Cretaceous volcanics, serpentines and other rock types[12], which seems to indicate the existence of Cretaceous igneous activity.

Comparison with other continental magnetic lineations

Magnetic anomalies in the Primorye and Sikhote-Alin provinces of eastern Asia show a distinct correlation to the belts of intrusions of intermediate to ultrabasic compositions[13]. The two eastern lineations (Fig. 1 v and vi) are located at both sides of the Sikhote-Alin granitic massif. One of the lineations, which runs along the coast of eastern Primorye and extends over the continental shelf on the Japan Sea side, is a coupled belt comprising a negative belt on the west and a positive belt on the east. Although the amplitudes of the magnetic anomalies are not reported in the Russian data, we judge, from the trend of the lineation (about N40°E), that along the coast of eastern Primorye lineation v is caused by a normally magnetised block, though the absence of negative anomaly belts associated with lineations vi and vii, which have the same trend, is not easily explicable. The close correlation between the ultramafic intrusive belts of eastern Asia and the magnetic lineations could indicate that the magnetic anomalies east of north-eastern Honshu are also caused by ultramafic intrusions. The orogenesis of the Sikhote-Alin Massif is supposed to have occurred during the Upper Cretaceous. Although the ages of the intrusive rocks around its sides are not reported, we think it likely that the Sikhote-Alin orogenesis is intimately related to the neighbouring intrusions (which therefore also occurred in the Upper Cretaceous or later). The sources of the magnetic lineations at Sakhalin and along the northern margin of the Kuril Basin are not known. We
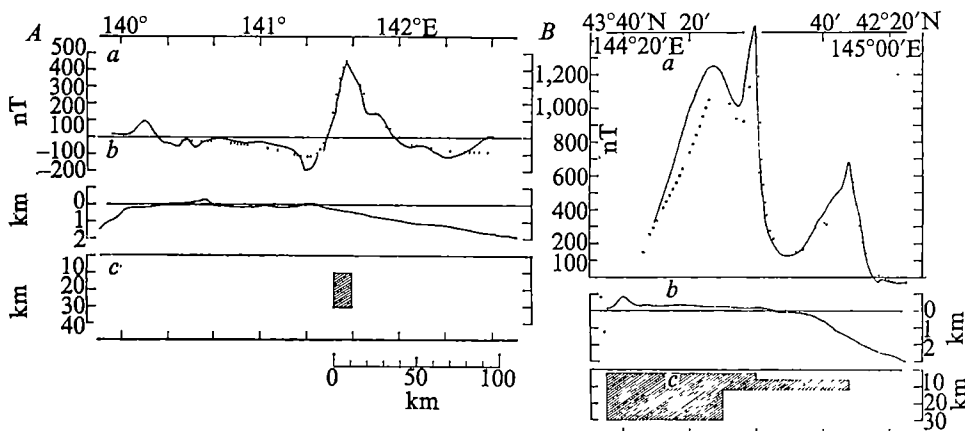
Fig. 2 Profiles of total magnetic intensity anomalies (a), bathymetries (b) and magnetisation models (c) across lineation i along line A and lineation ii along line B. The intensity of magnetisation is assumed to be 5×10^{-3} e.m.u. cm^{-3} for both cases. A: Present magnetic field—declination, N8°W; inclination, 53°; horizontal component, 28,500 nT. Magnetisation model—declination N45°W; inclination 53°. B: Present magnetic field—declination, N8°W; inclination, 56°; horizontal component, 27,200 nT. Magnetisation model—declination, N8°W; inclination, 56°.
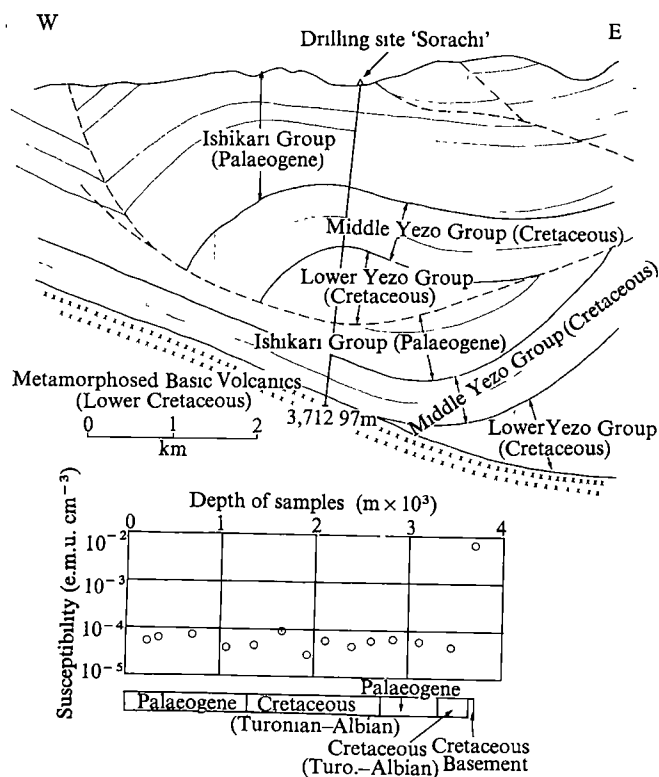
W   E

which it takes for a buried dyke to cool from 1,000 °C down to 500 °C indicates that about 10 Myr are needed[15]. Such a long cooling time together with the slower accumulation of intrusive materials suggests that at least several million years of steady magnetic polarity would be necessary before a continental intrusion can become magnetised in a fixed direction. Therefore, only two possible ages—Jurassic and Cretaceous—can be assigned to the intrusive belts. Thus, we think that most of the magnetisation which produced lineations i and ii may have been acquired during the long Cretaceous normal epoch, presumably between the Aptian and the Albian. On the other hand, the intrusions found at Primorye, which look somewhat younger than those of Honshu, may have been magnetised during the Cenomanian–Turonian. It is interesting that if this hypothesis is correct, the smooth magnetic zones of the oceanic basins correspond to the most magnetically anomalous areas of the continents.

assume, however, that they are all caused by basic or ultramafic intrusions.

Dating of the continental magnetic lineations can be attempted using the magnetic reversal time scale. The Mesozoic magnetic reversals[14] include two epochs during which the magnetic polarity remained normal for a long time: the first occurred before the Kimmeridgian (148 Myr BP); the second during the Aptian–Comacian (111.5–84.5 Myr BP). As remanent magnetisation is produced only when magma has cooled sufficiently, a steady remanent magnetisation cannot be acquired by continental intrusives (which seem to accumulate much more slowly than oceanic intrusives) during rapid magnetic reversals. Moreover, an estimation of the period

Inference of the movement of the island arcs

The openings of the Japan Sea and the Kuril Basin are inferred to have occurred during the Tertiary. As it is generally accepted that the formation of island arcs is the result of active volcanism caused by the descent of an oceanic plate[16], a tracing of the history of the volcanism and/or the occurrence of the magnetic lineations could reveal the evolution of island arc formation. Unlike the oceanic magnetic lineations, the continental magnetic lineations are not necessarily aligned according to their ages. It seems probable, however, that the igneous activity responsible for the magnetic lineations occurred at closer spacings than are now recognised: for example, we suggest that the Lower Cretaceous intrusion east of north-eastern Honshu must have been closer to the outer margin of eastern Primorye, where another continental
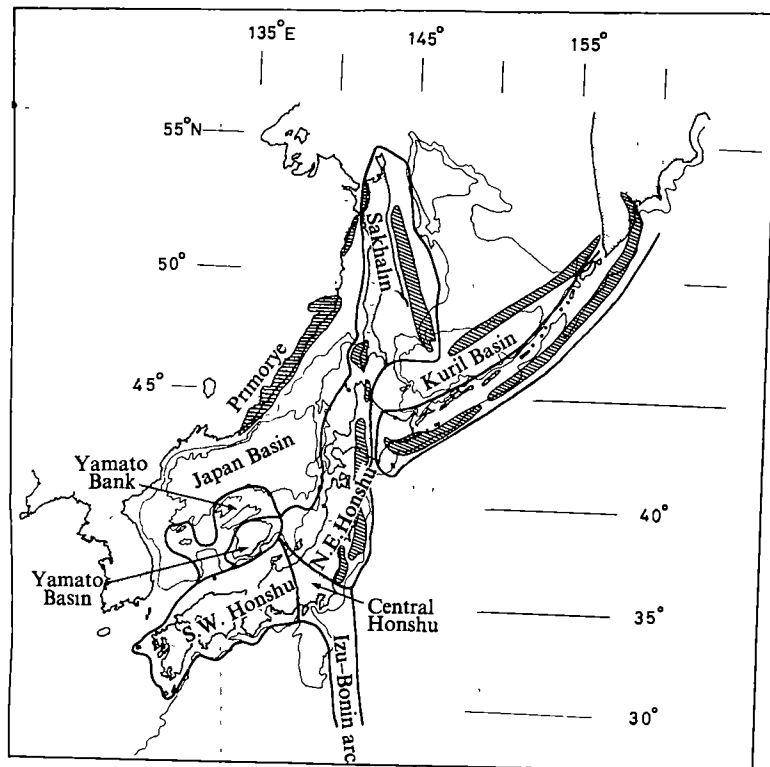
Fig. 4 Boundaries (thick solid lines) showing crustal blocks of Japan, Kuril and Sakhalin provinces. Hatched areas, marked magnetic lineations.
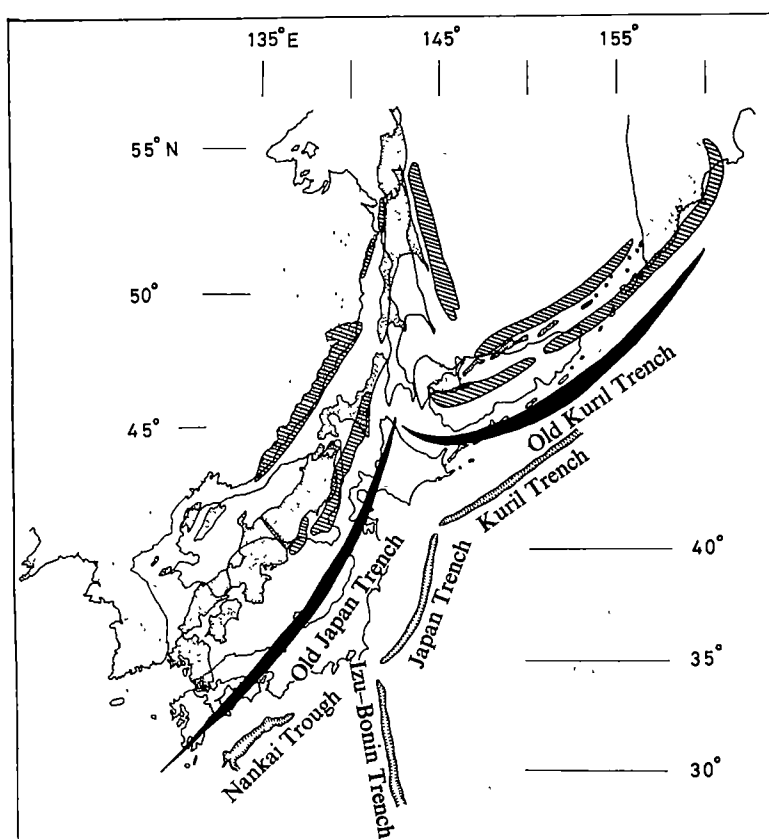
Fig. 5 Reconstruction of a possible feature of the Japan–Sakhalin–Kuril arcs before the Tertiary. Dotted areas, old continent and islands; hatched areas, marked magnetic lineations; Broad solid areas, possible locations of the old Japan and the Kuril trenches.

magnetic lineation is observed. The locations of the volcanic belts may be affected[17] by both the location of the subduction zones and the dip of the descending plates, resulting in a disorderly configuration of the volcanic–orogenic belts with time. We think, however, that the distances between successive (Cordilleran-type) orogenic belts observed at the continental margins are, in most cases, 200–300 km. If this should hold for general cases, the distance of more than 800 km at present recognised between the massifs of northeastern Honshu and the Sikhote-Alin suggests a more recent detachment of Honshu from the eastern Asiatic mainland; that is, a later opening of the Japan Sea. Although this argument requires no magnetic evidence, the documentation of magnetic lineations and their sources has enabled us to generalise the argument to apply to marine areas.

Figure 4 shows the tentative boundaries of continental blocks into which the island arcs can be divided and reconstructed so as to form the eastern Asiatic mainland at a time before the Tertiary. We have used our own criteria of reconstruction.

● We regard parts of an island arc with continental magnetic lineations as a single crustal block.

● Marginal basins such as the Japan Basin, the Yamato Basin and the Kuril Basin are replaced by continental crust.

● The Yamato Bank is regarded as continental crust.

● The central portion of Honshu together with the Izu-Bonin Ridge are excluded because they are very young.

● The Honshu, the western part of Hokkaido, and Sakhalin are treated as a single block because they seem to be connected across the Tsugaru and the Soya Straits, as is verified by seismic[18] and gravity results[19].

● The eastern part of Hokkaido is connected to the block of the Kuril Arc.

● Each block is either translated or rotated in such a way that the continental magnetic lineations become parallel and as close to each other as possible. In this case, north-eastern Honshu turns clockwise, whereas south-western Honshu turns anticlockwise.

Figure 5 shows a reconstruction for the Japan–Sakhalin–Kuril–Kamchatka region. A most significant corollary has been obtained as a result of dividing the Hokkaido district into western and eastern parts. The Hidaka Massif, trending north–south in the middle of Hokkaido, is known as a unique collision-type zone[20] showing a reversed, paired metamorphic belt[21], and it may be accounted for in terms of the collision of two island arcs. The area west of the Hidaka Mountain, that is, the Ishikari zone, is one of the most highly fractured and folded zones in Japan. As seen from the results of the test drilling at the Sorachi region (Fig. 3), the eastern Palaeogene sedimentary layers step over the western portions of the same layers. This suggests that intense folding and fracturing occurred in and after the Palaeogene. Results from seismic refraction surveys (S. Asano *et al.*, unpublished), P-wave travel-time anomalies (T. Moriya *et al.*, unpublished) and electrical conductivity anomalies (Y. Nishida, unpublished) also indicate abrupt changes of crustal thicknesses and depths of conductive layers in the mantle between western and eastern Hokkaido. This evidence suggests that the Hidaka Massif was built when the eastern crust of Hokkaido rode over the western crust during a collision of two island arcs, when the Honshu–Hokkaido–Sakhalin block was drifting south-eastwards, away from eastern Asia, and the Kuril Arc was moving southwards. A convergence of crusts occurred at the present Hidaka Mountain area. The reversed paired metamorphism of the Hidaka region may be regarded as a result of the upheaval and exposure of the crust on the eastern side, which was produced by the igneous activity of the former Kuril subduction zone.

We thank Dr Katsuro Ogawa of the Japan Petroleum Development Corporation for his advice.

1 Oshima, S., Kondo, T., Tsukamoto, T., and Onodera, K., *Rep Hydrogr Res* (Publication from the Japan Hydrographic Office, Tokyo), 10, 39–44 (1975)
2 Geological Survey of Japan, *Total intensity aeromagnetic maps* (1972, 1973, 1974).
3 Demenitskaya, R. M., *Crust and Mantle of the Earth*, 280 (Nedra Publishers, 1967).
4 Krasniy, M. L., *Proc. first Japan–USSR Symposium on Solid Earth Sciences* (Far East Publishers, Sakhalin Province, 1972).

5 Uyeda, S., *et al*, *Bull. Earthq. Res. Inst.*, 45, 799–814 (1967)
6 Sugimura, A., Matsuda, T., Chinzei, K., and Nakamura, K., *Bull. Volcan.*, 26, 125–140 (1963)
7 Kawai, N., Nakajima, T., and Hirooka, K., *J. Geomag., Geoelect.*, Tokyo, 23, 269–293 (1971).
8 Petroleum Development Society of Japan, *Present status of Japanese petroleum mining industry and technology*, Publication commemorating the 40th anniversary of the society (Lattice Publishers, Tokyo, 1973).
9 Saito, T., Watanabe, S., and Ono, Y., Abstract for the Fall Annual Meeting of the Society of Japan Mining Industry (Society of Japan Mining Industry, Tokyo, 1971).
10 Kametani, T., and Yoshimura, Y., Butsuri-Tanko (Geophysical Exploration), 17, 72–81 (1964).
11 Yamada, N., *Earth Sci.*, Tokyo, 85, 53–58 (1966).

12 Geological Survey of Japan, Geological Map of Japan, fourth edition (1971).
13 Kulnitch, R. G., *Proc. first Japan–USSR Symposium on Solid Earth Sciences*, 33–39 (Far East Publishers, Sakhalin Province, 1972).
14 Larson, R. L., and Pitman, W. C. III, *Bull. geol. Soc. Am.*, 83, 3645–3662 (1972).
15 Horai, K., *J. geophys. Res.*, 79, 1640–1646 (1974).
16 Uyeda, S., and Miyashiro, A., *Bull. geol. Soc. Am.*, 85, 1159–1170 (1974).
17 Katili, J. A., *Coordination Committee for Offshore Prospecting Technical Bulletin*, 7, 23–37 (1973).
18 Zverev, S. M., *Proc. first Japan–USSR Symposium on Solid Earth Sciences*, 110–115 (Far East Publishers Sakhalin Province, 1972).
19 Tomoda, Y., Segawa, J., and Tokuhiro, A, *Proc. Japan Acad.*, 46, 1006–1010 (1970)
20 Horikoshi, E., *Kagaku*, 42, 665–673 (1972).
21 Miyashiro, A., *J. Petrol.*, 2, 277 (1961).

Growth inhibition of animal cells by succinylated concanavalin A

Raphael J. Mannino & Max M. Burger

Department of Biochemistry, Biocenter, University of Basel, Klingelbergstrasse 70, CH 4056 Basel, Switzerland

A non-agglutinating lectin preparation, succinyl-con A, inhibits the growth of untransformed 3T3 mouse fibroblasts in a manner that mimics density-dependent growth inhibition. The growth inhibitory interaction between succinyl-con A and cells occurs exclusively during the mitotic and/or early G_1 phase of the cell cycle.

A DIRECT role for the cell surface in the maintenance and regulation of cell growth has been suggested but never convincingly demonstrated. Lectins which bind sugars have been successfully used to examine the composition and properties of that part of the cell surface that is available to the extracellular environment. In a number of cell types a correlation has been shown between saturation density in tissue culture and both agglutinability with lectins[1] and, in defined conditions, tumorigenicity[2]. Several investigations have demonstrated a correlation between the agglutinable surface configuration and growth of both normal and transformed cells[3].

We have reported previously that normal cells during mitosis also exhibit the agglutinable surface configuration and have suggested that this mitotic surface change might be involved in growth regulation[4]. In this report we present evidence that a non-toxic, non-agglutinating lectin preparation interacts with 3T3 mouse fibroblasts during the mitotic phase of the cell cycle and induces a "density-dependent like" growth inhibition.

Growth of 3T3 cells inhibited by succinyl-con A

The enzymatic preparation of non-agglutinating concanavalin A (con A) by protease[5,6] has proved difficult (although a detailed preparative procedure has been described by Evans and Jones[7]) as compared with the succinylation procedure of Gunther *et al.*[8]. A slight modification of that procedure, that is, scaling up the preparation sixfold and introducing affinity chromatography on Sephadex G-50 after the final succinylation, has reduced the contamination by tetrameric con A from approximately 5% to less than 0.1% when assayed by sedimentation in the analytical ultracentrifuge and glycogen precipitation. There were no detectable differences in the growth inhibitory properties of these various succinyl-con A preparations.

Succinyl-con A inhibits the growth of untransformed 3T3 cells in a way that depends on concentration (Fig. 1*a*). The growth inhibition seems to be specific for succinyl-con A since as much as 500 µg ml⁻¹ succinylated bovine serum albumin (succinyl-BSA) has no inhibitory effect on cell growth and, furthermore, since growth inhibition by 500 µg ml⁻¹ succinyl-con A can be prevented (95% that of control) by

10^{-2} M α-methyl-D-mannoside, a hapten specific for con A. Succinyl-con A inhibited cells accumulate in the G_1 phase of the cell cycle (determined by impulse cytophotometry) and show a rate of ³H-thymidine incorporation per cell identical to that of a density inhibited monolayer.

Other experiments suggest that succinyl-con A does not inhibit growth by binding to, and therefore "removing" from the medium a necessary growth factor, but rather that succinyl-con A interacts directly with the cell. First, Dulbecco's modified Eagle's medium preincubated with 500 µg ml⁻¹ succinyl-con A at 37 °C for 24 h, and then filtered by diaflow ultracentrifugation to remove the succinyl-con A before the addition of calf serum, supported growth to the same level as untreated medium. Second, an increase in the serum concentration of the growth medium results in an increase in the final density of both control and succinyl-con A–treated cells. Significantly, however, the percentage of growth inhibition at a given concentration of succinyl-con A is independent of the serum concentration.

Inhibition of growth by succinyl-con A seems to be non-toxic since it is reversible simply by removing the medium containing the succinyl-con A and replacing with fresh medium (Fig. 1*b*). Succinyl-con A–inhibited cells show the same percentage of cells responding to serum pulses with succinyl-con A as control cells show to serum pulses without succinyl-con A. The plating efficiency of cells inhibited by succinyl-con A is similar to that of cells from a density-inhibited monolayer.

It should be noted that although quantitative differences in the growth inhibitory capacity of succinyl-con A can vary as much as twofold when using different cell clones and different succinyl-con A preparations, the qualitative effects are consistent. The data presented here reflect some of our least active succinyl-con A preparations. Some preparations decrease saturation densities to 50% with 200 µg ml⁻¹ succinyl-con A.

Inhibition of growth is dependent on cell density

An interesting aspect of growth inhibition induced by succinyl-con A is that the cell density at which growth ceases is independent of the initial, that is, plating, density (Table 1). It seems that growth inhibition is some function of both succinyl-con A–cell interactions and cell–cell interactions and that succinyl-con A–cell interactions and cell–cell interactions are similar effects which in some way can substitute for one another to inhibit growth.

Succinyl-con A acts only during mitosis and/or early G_1

Synchronously growing 3T3 cells were tested for their susceptibility to succinyl-con A at various times during the

cell cycle (Fig. 2). Even though succinyl-con A may be present from the initiation of a new cell cycle in G_0, through the rest of G_1, S, and G_2, one round of cell division is not inhibited; the cells go through mitosis and come to rest in G_1 (Fig. 2a). Indeed, if succinyl-con A is removed just before mitosis, the cells continue to grow, going through a second round of the cell cycle essentially as if they had never been in the presence of succinyl-con A; succinyl-con A added immediately before the cells enter mitosis, however, causes a cessation of growth following the completion of that mitotic phase.

By adding succinyl-con A to a synchronously growing population of 3T3 cells at various times throughout the mitotic period, it was possible to obtain a more detailed understanding of the inhibition induced by succinyl-con A

Fig. 2 Cell cycle dependence of succinyl-con A–induced growth inhibition. a, 3T3 cells were subcultured into DME + 2% calf serum. When growth had become stationary[37], the medium was replaced with DME + 10% calf serum ± succinyl-con A (500 μg ml⁻¹) and replaced again 23 h later, just before mitosis: ●, control; ■, succinyl-con A at the first medium change, none at the second; □, succinyl-con A at the second medium change; ○, succinyl-con A at both medium changes. b, 3T3 cells from a 2-d-old confluent monolayer were subcultured into DME + 10% calf serum; succinyl-con A (final concentration 500 μg ml⁻¹) was added at various times during the mitotic period; cells at a density indicated by the continuous line (lower curve) at the time of addition of succinyl-con A grew to a corresponding final density indicated by the broken line (upper curve); each point represents three samples counted in duplicate; cell numbers were determined as described in Fig. 1.

Fig. 1 Characteristics of succinyl-con A–induced growth inhibition of 3T3 mouse fibroblasts. 3T3 cells were subcultured into 3.5 cm tissue culture dishes containing Dulbecco's modified Eagle's medium (DME) + 10% calf serum; the medium was changed after 1 and 4 days of growth to DME + 5% calf serum ± succinyl-con A as indicated. Cell numbers were determined using a Coulter counter after removal with a buffered EDTA (0.54 M)-trypsin (0.05%) solution; each point represents duplicate samples counted twice. a, Concentration dependence of succinyl-con A–induced growth inhibition; b, reversibility of growth inhibition. □, control; ○, 250 μg ml⁻¹ succinyl-con A; △, 500 μg ml⁻¹ succinyl-con A; ● and ▲, succinyl-con A removed on d 4 and replaced by fresh medium containing 5% calf serum.

Table 1 Density dependence of succinyl-con A–induced growth inhibition

Initial density (cells cm⁻² × 10⁻⁴)	Final density (cells cm⁻² × 10⁻⁴)	% of control
a Controls (0.15–0.50)	2.80±5%	
0.15	1.90	67.7
0.35	2.01	71.9
0.50	2.14	76.4
b Controls (0.18–0.51)	3.40±5%	
0.18	2.27	66.8
0.35	2.44	71.8
0.51	2.45	72.1

Experimental conditions were as described in Fig. 1; a, Cells grown in DME+4% calf serum +250 μg ml⁻¹ succinyl-con A; b, cells grown in DME + 5% calf serum + 250 μg ml⁻¹ succinyl-con A.

(Fig. 2b). As would be predicted in these conditions if the sensitive period of the cell cycle is mitotis and/or nearly G_1, the final density of a cell population is higher if more cells are allowed to pass through mitosis before the addition of succinyl-con A. The new information obtained from this experiment is that the midpoint of the "final density" curve (upper curve, Fig. 2b) is shifted to a time in the cell cycle that is approximately 1.5 h later than the midpoint of the "mitosis" curve (lower curve, Fig. 2b)—in three separate experiments, each done in triplicate, the shift was between 1.5 and 2 h. These data suggest that the phase of the cell cycle during which cells are sensitive to the inhibitory effects of succinyl-con A may be early in G_1, a time during which previously rounded cells, which have just completed mitosis, are returning to a flat morphology but still retain the "agglutinable" cell surface configuration.

to inhibit the growth of transformed cells by interacting with the cell surface. Two preliminary experiments using succinyl-con A covalently linked to polyacrylamide beads inhibited the growth of 3T3 cells to at least 30%.

Trowbridge and Hilborn[32] have reported that succinyl-con A binding to nearly confluent monolayers of 3T3 and SV3T3 cells is saturated at a concentration of 100 µg ml^{-1} at 0 °C in phosphate buffered saline, and they concluded that this concentration has no appreciable effect on cell growth. A closer examination of their data, however, seems to indicate approximately a 30% inhibition of 3T3 cell growth at 100 µg succinyl-con A ml^{-1}, an inhibition which can be reversed by 50 mM α-methyl-D-mannoside. Our findings that concentrations of succinyl-con A higher than 100 µg ml^{-1} have a marked, specific and non-toxic effect on the growth of 3T3 cells, may not be contradictory to the binding studies of

---

Table 2 Summary of surface characteristics of interphase, mitotic and transformed cells

Surface features*	Interphase cells	Mitotic (and early G_1) cells	Transformed cells
Morphology—scanning electron microscope	Flat and relatively smooth surface	Round with many surface microvilli[18]	Less flat with irregular surface features including microvilli, blebs and/or ruffles[9]
Ease of detachment from substratum	Difficult	Easy	Easy
Electrophoretic mobility	Low	High[13]	High[11,12]
Lectins			
(a) Agglutinability[14]	(a) Low	(a) High	(a) High
(b) Binding of fluorescent labelled lectin[4,15]	(b) Low	(b) Higher	(b) Higher (only some)
Glycoproteins	Completely glycosylated	More fucose and sialic acid containing glycopeptides[17]	Incompletely glycosylated. More fucose and sialic[16] acid containing glycopeptides
Glycolipids			
(a) Forssman antigen reactivity	(a) Low	(a) High[19]	(a) High[18]
(b) Gangliosides (for reviews see refs 3 and 23)	(b) Completely glycosylated or "higher" gangliosides	(b) Not determined	(b) Reduction of "higher" gangliosides—accumulation of precursors
Cell contact dependence[20] of surface galactosyl-transferase activity	Dependent	Independent	Independent
Hynes protein	High levels	Very low levels[22]	Absent[21]
Surface antigens (for reviews see refs 3 and 23)	Relatively few	New antigens appear—some disappear	New antigens appear—some disappear
Virus induced fusion	Low	High[24]	Not determined
Surface bound heparin sulphate	High	Released into medium[26] Low	Low[25]

*Many of these characteristics have been determined for a single or at best only a few cell types. Also, when a particular characteristic has been examined in many cell types the surface changes indicated in this table have at times not been consistently observed.

Implications for growth control

Earlier, using lectins as probes for the examination of the cell surface, we suggested that the cell surface architecture of mitotic cells is similar to that of transformed cells and that this surface configuration might have relevance to growth control[4]. Recent data from several laboratories[3,4,9–26], summarised in Table 2, indicate in greater detail the striking similarity between the surfaces of mitotic and transformed cells. Such a pronounced correlation alone entices one to speculate about a cell surface involvement in the mechanisms regulating cellular proliferation.

There is much circumstantial evidence which suggests that succinyl-con A is exerting its inhibitory effect through specific binding to the cell surface. The vast majority of processes mediated by lectins, for example, receptor site patching and capping[27], cell agglutination, and lymphocyte mitogenesis[28,29], involve the binding of the lectin to the cell surface. Two other preparations, glycolipids obtained from *Salmonella minnesota* R mutants[30] and dextran sulphate polymers[31], have been reported

Trowbridge and Hilborn[32], since we believe that the binding that is important for growth inhibition occurs during mitosis and in culture conditions. Glucose and serum components bind competitively to succinyl-con A (M.M.B., unpublished) requiring, therefore, higher overall concentrations to reach the effective concentration at the cell surface. It is not surprising that the concentration dependence of succinyl-con A-induced growth inhibition does not agree with studies of con A or succinyl-con A binding.

We have also observed that succinyl-con A can reduce the growth of both polyoma and SV101 transformed 3T3 cells. Because the stimulatory effect of serum on cell growth is antagonistic to the inhibitory effect of succinyl-con A, inhibition of transformed cells induced by succinyl-con A is best observed in the presence of low serum concentrations (2–5%). In these conditions we have observed a marked, non-toxic reduction in the growth rate of these transformed cell lines, although we have not yet observed complete inhibition.

Some of the factors regulating the growth of 3T3 cells are shown in Fig. 3. On entering the mitotic phase of the cell

cycle the cells undergo a change in their surface architecture and receive a "go" signal which sends them into another round of the cell cycle. It is possible that a change in the intracellular level of cyclic nucleotides plays a part in this "go" signal[33], but, since there is no convincing evidence that cyclic AMP or cyclic GMP alterations are causally related to growth inhibition and control, many other mechanisms will have to be considered.
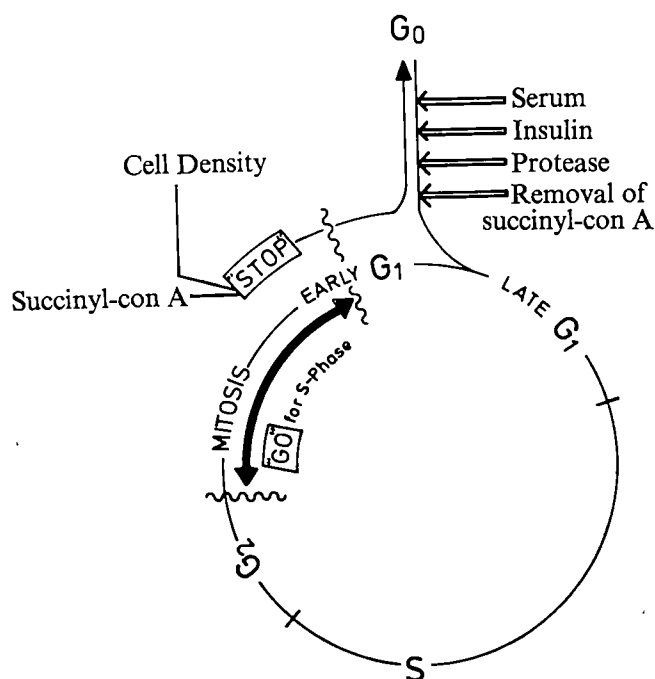
Fig. 3 Proposed interrelationships of various factors regulating the growth of 3T3 cells and the involvement of the mitotic cell surface configuration. The "go" signal occurs early in mitosis, perhaps concomitant with the change to the agglutinable state; the "stop" signal must be received before the cells revert back to the non-agglutinable state some time early in G_1. ←→ indicates the period of the cell cycle during which the cells are agglutinable.

When the cell density in the immediate environment of a given cell reaches a certain level, growth stops and the cell accumulates in G_0, a specific part of G_1. Pardee[34] has demonstrated that cells whose growth has been inhibited by high cyclic AMP levels, serum starvation or nutrient starvation, all accumulate at the same point in the cell cycle through a "restriction point" control mechanism. We would hypothesise that cells inhibited by succinyl-con A accumulate at the same point in the cell cycle by the same mechanism.

Cells that have stopped growing and are resting in G_0 can be induced to re-enter the growth cycle by a number of treatments, including serum stimulation, insulin treatment, urea treatment, mild proteolysis and, in the case of succinyl-con A-inhibited cells, removal of succinyl-con A. Serum stimulation, insulin treatment, urea treatment and mild proteolysis all cause the surface membrane to undergo very rapid change to the agglutinable state (Bombik, Bechtel, and M. M. Burger, unpublished, and Weston and Hendricks[35]). If succinyl-con A is added concomitantly with a serum pulse, cell division is not inhibited, even though the agglutinable surface configuration is known to be present (data not shown). This suggests to us that not only is a specific cell surface configuration required for succinyl-con A to exert its inhibitory effect, but also that the particular surface requirements must occur during a specific part of the cell cycle. We suggest therefore that cells are capable of receiving a "stop" signal

only in early G_1, when untransformed cells still exhibit the cell surface configuration detectable by lectin. If growing cells proceed through this period in the cell cycle without receiving a "stop" signal, they are committed both to a complete round of DNA replication and to cell division. Cells which do not receive the "stop" signal accumulate in G_0 but are "past the point of no return". If G_0 cells are stimulated to re-enter the growth cycle, they are unable to receive a "stop" signal, either from succinyl-con A or from high cell density, until they have proceeded through the entire cell cycle, entered mitosis and once again assumed an agglutinable surface configuration.

The biochemical nature of this "stop" signal is unknown, but two distinct types of mechanisms immediately present themselves. First, succinyl-con A could interact with the cell surface producing a generalised membrane effect, that is, an overall change in the cell surface membrane. Stoker[36] has suggested that density-dependent inhibition of growth is the result of a diffusion barrier established when cells grow to high densities; the interaction of succinyl-con A with the cell surface could establish a similar barrier to the diffusion or transport of metabolites essential to growth. Alternatively, succinyl-con A may increase cell surface "stickiness" leading to a decreased mobility after cell–cell collisions and this decreased mobility resulting in growth inhibition (there is no evidence for an increase in the stickiness between cells and substratum).

The second possibility is that succinyl-con A interacts with a specific, unique surface receptor, triggering a series of biochemical events which result in the production of a growth inhibitory "second messenger".

Our data suggest that succinyl-con A interacts with untransformed 3T3 cells during the early G_1 phase of the cell cycle to inhibit growth by a mechanism that, by all the criteria measured so far, seems to be identical to density-dependent inhibition of growth.

1 Pollack, R., and Burger, M. M., *Proc. natn. Acad. Sci U S A.*, 62, 1074–1076 (1969)
2 Aaronson, S., and Todaro, G., *Science*, 162, 1024–1026 (1968).
3 Rapin, A. M., and Burger, M. M , *Adv. Cancer Res.*, 20, 1–91 (1974).
4 Fox, T., Sheppard, J., and Burger, M. M , *Proc natn. Acad Sci. U S.A* , 68, 244–247 (1971)
5 Burger, M M , and Noonan, K , *Nature*, 228, 512–515 (1970).
6 Steinberg, B. M. S , and Gepner, I. A , *Nature new Biol.*, 241, 249–251 (1973).
7 Evans, P., and Jones, B., *Expl Cell Res* , 88, 56–62 (1974)
8 Gunther, G , Wang, J., Yahara, I , Cunningham, B , and Edelman, G , *Proc natn Acad. Sci. U S A* , 70, 1012–1016 (1973)
9 Porter, K. R , Todaro, G. J., and Fonte, V., *J. Cell Biol* , 59, 633–642 (1973).
10 Porter, K , Prescott, D., and Frye, J., *J. Cell Biol* , 57, 815–836 (1973).
11 Ambrose, E. J , James, A. M., and Lowick, J H B , *Nature*, 177, 576–577 (1956).
12 Ruhenstroth-Bauer, G , in *Cell Electrophoresis* (edit. by Ambrose, E. J), 66 (Churchill, London, 1965).
13 Kraemer, P M , *J Cell Biol* , 33, 197–200 (1967).
14 Noonan, K., and Burger, M. M , *Expl Cell Res* , 80, 405–414 (1973).
15 Turner, R. S , and Burger, M. M , *Ann. N Y. Acad Sci* , 234, 332–347 (1974).
16 Warren, L , Fuhrer, J. P , and Buck, C. A , *Fedn Proc.*, 32, 80–85 (1973)
17 Glick, M C , and Buck, C A , *Biochemistry*, 12, 85–90 (1973)
18 Hakamori, S , Gahmberg, C G., Laine, R., and Kijimoto, S , in *Control of Proliferation in Animal Cells* (edit. by Clarkson, B , and Baserga, R) 461–471 (Cold Spring Harbor, New York, 1974)
19 Wolf, B., and Robbins, P , in *Control of Proliferation in Animal Cells* (edited by Clarkson, B), and Baserga, R., 473–479(Cold Spring Harbor, New York, 1974).
20 Webb, G. C , and Roth, S , *J. Cell Biol* , 63, 796–805 (1974).
21 Hynes, R O , *Proc natn Acad Sci U.S A.*, 70, 3170–3174 (1973).
22 Hynes, R , and Bye, J , *Cell*, 3, 113–120 (1974).
23 Burger, M M., *Curr Top. cell. Regulation*, 3, 135–193 (1973).
24 Stadler, J K., and Adelberg, E. A , *Proc natn Acad Sci U S A* , 69, 1929–1933 (1972).
25 Chiarugi, V., Vannucchi, S., and Urbano, P , *Biochim. biophys. Acta*, 345,283–293 (1974).
26 Kraemer, P. M , and Tobey, R. A , *J. Cell Biol* , 55, 713–717 (1972)
27 Edelman, G., Yahara, I , and Wang, J., *Proc natn Acad Sci U S A* , 70, 1442–1446 (1973).
28 Powell, A , and Leon, M., *Expl Cell Res* , 62, 315–325 (1970)
29 Novogrodsky, A , and Katchalski, E , *Biochim biophys. Acta*, 228, 579–583 (1971).
30 Braslovsky, C., Trudel, M , Lallier, R , and Nigam, V., *J Cell Biol.*, 57 124–132 (1973).
31 Goto, M., Kataoka, Y., and Sato, H., *Gann*, 63 ,371–374 (1972)
32 Trowbridge, I , and Hilborn, D , *Nature*, 250, 304–307 (1974)
33 Moens, W , Vokaer, A , and Kram, R , *Proc. natn. Acad Sci U S A* ,72, 1063–1067 (1975)
34 Pardee, A , *Proc natn. Acad. Sci. U.S A* , 71, 1286–1290 (1974)
35 Weston, J , and Hendricks, K , *Proc. natn Acad Sci U.S A* , 69, 3727–3731 (1972).
36 Stoker, M , *Nature*, 246, 200–203 (1973)
37 Clarke, G , Stoker, M., Ludlow, A., and Thornton, M., *Nature*, 227, 798–801 (1970).

letters to nature

Massive black holes in globular clusters?

FOUR X-ray sources have been identified[1,2] with globular star clusters (NGC1851, NGC6441, NGC6624 and M15). These are the first X-ray sources to be optically identified with the oldest component (age $\sim 10^{10}$ yr) of the stellar population of our Galaxy; previous identifications of galactic X-ray sources were mostly with bright, young, Population I stars or with relatively recent supernova remnants. Katz[3], Clark[4] and Canizares and Neighbours[5] have discussed the apparent differences in frequency and population between the globular cluster X-ray sources and other galactic X-ray sources. They have assumed that the X-ray sources in globular clusters are, like the sources identified with Population I stars, binary stars in which a normal companion transfers mass on to a collapsed star, thereby producing X rays.

Here we suggest that the globular cluster X-ray sources may be a very different phenomenon from the previously studied binary X-ray sources. In particular, we suggest that the observed X-ray emission in globular clusters may be caused by accretion of gas on to a massive black hole at the centre of the cluster. This suggestion is consistent with theories of stellar and cluster evolution and with previous observations of globular clusters. Both the theory of cluster core collapse and some observational consequences of black holes in globular clusters have been widely discussed (see refs 6–8 and references therein).

We assume a central massive object accreting at the rate $\dot{M}_{acc}$ and converting rest mass to X rays (2–10 keV) with efficiency ε as follows:

$$L_x = (\varepsilon/0.1)\,(\dot{M}_{acc}/10^{-9}M_\odot/\text{yr}) \times 10^{37}\ \text{erg s}^{-1} \qquad (1)$$

We have considered two possible sources of accretional mass. Normal stellar evolution in an old system will release

$$\dot{M}_{gas} \approx 10^{-11} M_\odot\ \text{yr}^{-1}\,(L_{cl}/L_\odot) \qquad (2)$$

or about $10^{-6} M_\odot\ \text{yr}^{-1}$ (with an uncertainty of at least an order of magnitude) in the entire cluster. If this gas is produced

Fig. 1 Globular cluster central escape velocity and central relaxation time from data of Peterson and King[10]. Circled points are known X-ray sources. Arrow indicates change for M15 if its core radius has been overestimated by a factor of 1.5. Clusters above hatched regions are expected to be tightly enough bound to retain gas.
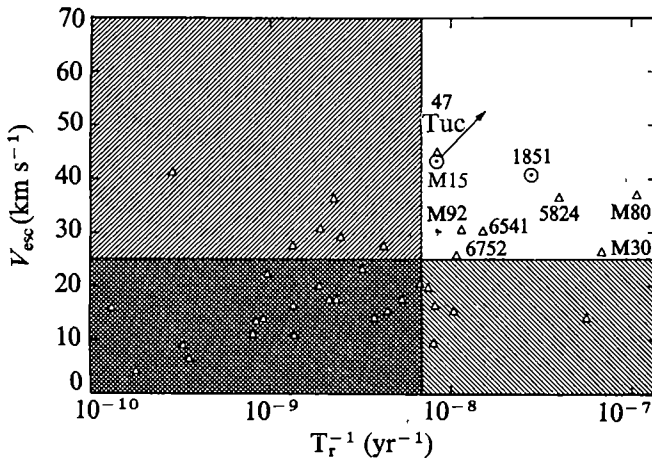
with a velocity of 25 km s^{-1} (characteristic of planetary nebula expansion rates) it will be retained in some, but not all, of the globular clusters (Fig. 1). Dimensional arguments suggest[9] that $T \geq T_{BB} \equiv [GMM_{acc}/8\pi R^3\sigma]^{\frac{1}{4}}$ (where R is the distance from the black hole), which implies that the X-ray efficiency could be appreciable. Massive black holes may also be able to acquire the gas required for accretion directly by tidally disrupting stars which approach too closely. A very rough analysis of this problem gives a rate proportional to $M_H^{4/3} \rho_c^{\frac{1}{2}} r_c^{-1}$ where M_H is the mass of the hole and (ρ_c, r_c) are the cluster core density and radius. Taking observed numbers for these parameters from Peterson and King[10] (with $|M/L|_{cluster}$ assumed 1), we find $\dot{M}_{dis} \lesssim 10^{-9}(M_{BH}/10^3 M_\odot)^{4/3} M_\odot$ yr^{-1}. This rate is much less than that given by normal stellar evolution but it is produced in the immediate vicinity of the hole and might be accreted with greater efficiency.

If the gas is streaming through the centre of the cluster with a characteristic velocity, v_{gas}, then it will be captured[11] at a characteristic radius

$$R_c = GM_{BH}/v_{gas}^2 \simeq 2 \times 10^{13}\ \text{cm}\ (M_{BH}/M_\odot)\,((25\ \text{km s}^{-1})/v_{gas})^2$$

where M_{BH} is the mass of the black hole. At somewhat larger distances from the black hole, the characteristic density would be

$$n_{gas} \sim (\pi R_c^2 v_{gas} m_{proton})^{-1}(dM/dt)_{accretion} \qquad (3)$$

or

$$n_{gas} \sim 15\ \text{cm}^{-3}(L_x/10^{37}\text{erg s}^{-1})(0.1/\varepsilon) \times \\ \times (10^3 M_\odot/M_{BH})^2 (v_{gas}/(25\ \text{km s}^{-1})^3$$

For a gas with this typical density, 15 cm^{-3}, the total mass, if it were spread uniformly over the central R_{pc} of the cluster, is only $M_{gas} \sim 2 M_\odot R_{pc}^3$. There may also be additional gas not in the cluster core, part of which will eventually fall into the cluster centre.

There is a minimum black hole mass that can produce the observed X-ray intensity, since the mass of gas accumulated in the cluster is less than or equal to $\dot{M}_{gas} t_{encounter}$, where $t_{encounter}$ is the time since the last passage through the galactic disk. The accretion rate, $\dot{M}_{accr} = 4\pi R_c^2 \rho_c v_c$, is proportional to M_{BH}^2 and must be equal to $(L_x/c^2\varepsilon)$. The pressure scale height for the gas heated by X rays is ~ 1 pc for typical parameters which implies that much of the gas is unavailable for accretion. Assuming an approximate isobaric relationship, $\rho_c T_c = \rho_{core} T_{core}$ (where the cluster core is typically ~ 0.4 pc) and inserting the maximum allowable value for ρ_{core} ($\sim 0.1 \dot{M}_{gas} t_{encounter}$/volume of the core) in equation (3), one finds a minimum black hole mass:

$$M_{BH} \geq 10^{2.5} M_\odot \left| \left(\frac{0.1}{\varepsilon}\right)\left(\frac{L_x}{10^{37}\text{erg s}^{-1}}\right)\left(\frac{v_{gas}}{25\ \text{km s}^{-1}}\right)^3 \left(\frac{T_{core}}{0.1 T_c}\right) \right|^{1/2}$$

(4)

Also because of the limited amount of gas available near the black hole, the X-ray luminosity is, for typical parameters, only $L_x \sim 10^{32.5}$ erg s^{-1} $(M_{BH}/M_\odot)^2 (M_{gas}/10 M_\odot)(\varepsilon/0.1)$, much less than the Eddington limit for the masses we consider (but

comparable with the observed luminosities). Normal cluster stars could have retained close white dwarf companions and accretion onto these might give $L_x \leq 10^{36}$ erg s^{-1}, but could not explain a bright source such as NGC6624.

Most of the gas will be ionised by ultraviolet radiation from the stars in the cluster[12] as well as by the X radiation from the vicinity of the black hole. The total mass of ionised gas may be too small to produce any observable absorption in the X-ray spectrum or to have been detected in the most sensitive radio searches conducted so far (typical good upper limits for H II are $\sim 30M_\odot$ for several clusters[13]; there are stronger limits[14,15] for H I). The most sensitive possible radio searches for thermal bremsstrahlung from H II gas in the X-ray clusters would be of interest.

We note that a search for Balmer emission lines in the cluster centres would also be of great interest. The expected total flux from the ionised gas is $\sim 2 \times 10^{-13}$ erg cm^{-2} s^{-1} (distance/ 10 kpc)$^{-2}$ in H_β. This is much less than the continuum optical emission from a typical X-ray globular cluster even when one takes into account only that small part of the cluster continuum that would lie within a reasonably small slit. Nevertheless one might, with good luck and excellent technique, be able to place significant limits on, or detect, the postulated gas. Typical expected equivalent widths are of the order of 10^{-2}Å in an H_β line of ~ 1 Å width and depend on, among other things, the slit size used and the unknown spatial extent of the ionised gas. The usually strong forbidden emission lines, such as O II λ3727 and O III λ5007, should be missing (at least near the centre of the cluster) because the typical ionisation stage in the presence of the X-ray flux would be higher.

The model considered here leads to two unambiguous predictions. First, the X-ray emission should be point-like and located at the centre of the globular cluster. Second, there should be no eclipses, orbital or spin periods observed in the X rays (although periods $\sim 0.1(M_{BH}/10^3M_\odot)$ s, from particles orbiting near the black hole, might occur in some circumstances). One can show that a pair of massive black holes, or a swarm of lower mass condensed objects, would have evolved dynamically to a single central rotating black hole through many-body interactions and gravitational radiation. One might hope that the observable star distribution and velocity dispersion near the centre of a globular cluster X-ray source are somewhat different than in other clusters. But for an integrable star density $\rho \propto r^{-\alpha}$ near the hole (Peebles[8] gives $\alpha = 9/4$), one finds a fractional increase in the luminosity of only

$$\Delta L/L_{core} \approx [\alpha/(3-\alpha)](M_{BH}/M_{core})^3 \qquad (5)$$

which is probably not observable unless $M_{BH} \leq 0.2M_{core}$. The detection[15] of 10.2 μm radiation from the centre region of M15 suggests that something unusual is indeed happening in the core of this X-ray cluster. Further infrared measurements of globular clusters are of the greatest possible interest (especially since a large fraction of the total luminosity in the X-ray clusters could be in this spectral region).

Finally we must consider the reasons why, on our model, only a small fraction ($\sim$ a few per cent) of the globular clusters in our Galaxy are X-ray sources. There are at least three obvious possibilities. First, only a moderate fraction of the clusters are bound tightly enough to retain gas. Second, the gas production rate may be sporadic. Third, it may be that only a few of the globular clusters have evolved dynamically far enough to have formed massive black holes in their centres. From a detailed investigation of Spitzer's Monte-Carlo simulation[6,7], it seems that core collapse occurs at a time $\tau \simeq 10^2\tau_c$ from the moment when the central relaxation time is measured to be τ_c. Using data from Peterson and King[10], we show the deduced values of τ_c in Fig. 1. The clusters to the right of the hatched region should collapse within the next 12×10^9 yr. They are correspondingly the best candidates for clusters

within which core collapse has already occurred. It is interesting to note that the two X-ray clusters (shown by circles in Fig. 1) in the well-studied group of Peterson and King lie in the relatively small region having short core relaxation time and large central escape velocity.

JOHN N. BAHCALL

Institute for Advanced Study

JEREMIAH P. OSTRIKER

Princeton University Observatory,
Princeton, New Jersey 08540

1 Giacconi, R , et al., Astrophys J. Suppl. No 237, 27, 37 (1974).
2 Clark, G W., Marhent, T H., and Li, F. K., Astrophys. J. Lett. (in the press).
3 Katz, J. L., Nature, 253, 698 (1975)
4 Clark, G. W , Astrophys J. Lett (in the press).
5 Canizares, C. R , and Neighbours, J E., Astrophys J Lett (in the press)
6 Spitzer, L., and Thuan, T X., Astrophys. J., 175, 31 (1972).
7 Ostriker, J. P., Spitzer, L., and Chevalier, R A., Astrophys. J Lett., 126, L51 (1972).
8 Peebles, P. J. E., Astrophys J., 178, 371 (1972).
9 Pringle, J. E., and Rees, M J., Astr. Astrophys , 21, 1 (1972).
10 Peterson, C. J., and King, I. R , Astrophys. J. (in the press).
11 Bondi, H , Mon. Not R. astr. Soc , 112, 195 (1952)
12 Minkowski, R., and Osterbrock, D. E., Astrophys. J , 129, 58 (1959)
13 Hills, J. G , and Klein, M. J., Astrophys. Lett , 13, 65 (1973)
14 Roberts, M S., Nature, 184, 1555 (1959).
15 Knapp, G R , Rose, W. K., and Kerr, F J , Astrophys. J , 186, 831 (1973).
16 MacGregor, A D., Phillips, J P , and Selby, M J , Mon. Not. R. astr Soc., 164, 31P–33P (1973)

Anisotropic redshift distribution for compact galaxies with absorption spectra

RUBIN et al.[1] have found that in one hemisphere of the sky ScI galaxies with $14.0 \leq m \leq 15.0$ have a mean velocity $1,365 \pm 200$ km s^{-1} larger than in the other hemisphere. Anisotropies, corresponding to this, have also been found for brightest cluster galaxies, supernovae (T.J., G. Le Denmat, M.M., J. C. Pecker and J.P.V., unpublished and ref. 2), and Markarian galaxies (H.K. and M.M., unpublished). Here we present a similar result for absorption line compact galaxies.

Zwicky's catalogue[3] has been used, selecting from it the compacts with measured redshift and absorption spectra. Since absorption line and emission line compact galaxies have different (m, z) relations these cannot be sampled together, and the number of the latter is rather small for a separate analysis. In absorption spectra also, those with [O II] λ 3727 emission were included in the case that the compact is not blue (most emission objects are blue), since this line is a common component in the normal spectrum of galaxies. Galaxies which are not compact but only have a compact core or some other compact part were not included, for the same reason as the emission objects. In the case of pairs only one component was included in order not to have double weight for these objects, a minority of the sample. The brighter one was taken if m_p is given for each component but if not we corrected m_T by $+ 0.75$ mag as an estimate of the individual m_p. If one of the components was indicated to be brighter than the other, a half correction, 0.37 mag, was applied to m_T. The mean velocity was used in the case of pairs. The cosec law with coefficient 0.25 mag was used for correction of galactic absorption, and galaxies with $|b^{II}| < 20°$ were excluded. The sky was divided into two regions, as was done by Rubin et al.[1] (Fig. 1). As in the earlier studies, the smallest velocities $V < 3,000$ km s^{-1} (only one compact) were not considered. The Hubble modulus $HM = \log V - 0.2m$ is the suitable parameter for this kind of study since it is purely observational and reflects best individual absolute luminosities or, alternatively, individual V/r values in a sample.
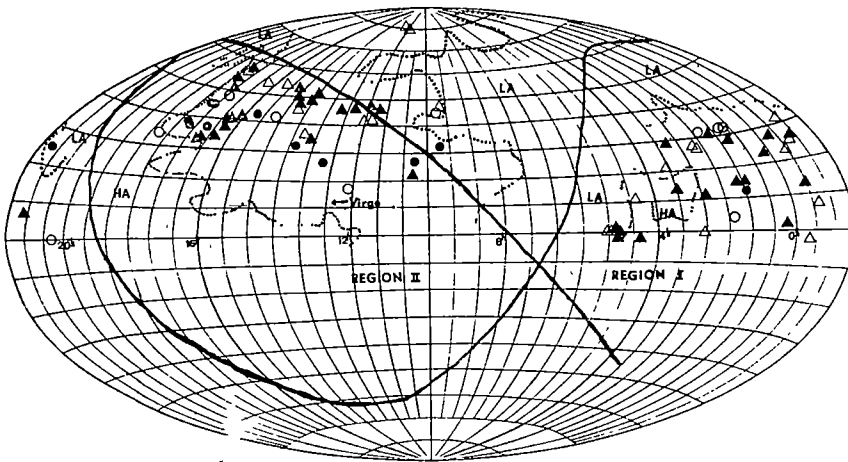
Fig. 1 Distribution of compact galaxies with known redshifts and absorption spectra on the sky. Circles are for galaxies with $m_c \leqslant 15.0$ and triangles for those with $m_c > 15.0$. Filled symbols show galaxies with $\delta HM > 0$ and open symbols those with $\delta HM < 0$ where $\delta HM = HM - \langle HM \rangle$, the mean value $\langle HM \rangle$ being calculated separately for intervals $m_c \leqslant 15.0$ and $m_c > 15$ 0. The full curve from upper left to lower right is the borderline between the two regions of sky. The other full curve shows the galactic equator. The areas included between dashed lines and the galactic equator are those of exceptionally high and low absorption, denoted by HA and LA, respectively. The latter have been transformed from the map in galactic coordinates given by Holmberg[4], and show areas where absorption deviates from the law $A = 0.25 \, \mathrm{cosec} |b^{II}|$ by ± 0.15 mag or more.

Considering the interval of corrected magnitude $14.0 < m_c \leqslant 15.0$, we obtain for the mean HM in region I the value $\langle HM \rangle_I = 0.88 \pm 0.10$ (5 galaxies) and for region II $\langle HM \rangle_{II} = 1.12 \pm 0.04$ (10 galaxies). For the galaxies with $m_c \leqslant 15.0$ (all have $m_c > 13.0$), $\langle HM \rangle_I = 0.93 \pm 0.07$ (7 galaxies) and $\langle HM \rangle_{II} = 1.10 \pm 0.04$ (15 galaxies). For the galaxies with $m_c > 15.0$ (all have $m_c < 18.0$), $\langle HM \rangle_I = 0.82 \pm 0.04$ (34 galaxies) and $\langle HM \rangle_{II} = 0.87 \pm 0.03$ (27 galaxies). For the whole interval $13.0 < m_c < 18.0$, $\langle HM \rangle_I = 0.84 \pm 0.04$ (41 galaxies) and $\langle HM \rangle_{II} = 0.95 \pm 0.03$ (42 galaxies). In the different m_c intervals the differences in $\langle HM \rangle$ between regions II and I are 0.24 ± 0.107, 0.17 ± 0.089, 0.05 ± 0.050 and 0.11 ± 0.050, respectively. These are significant at 2.24 σ, 1.91 σ, 1.00 σ, and 2.20 σ levels, corresponding to chance probabilities 0.020, 0.035, 0.170 and 0.015. The mean difference of velocities of objects in region II and I is $3,400 \pm 1,850$ km s^{-1} for $m_c < 15$ and $700 \pm 1,400$ km s^{-1} for $m_c > 15$. For $m_c < 15.0$ the average values $\langle m_c \rangle = 14.3$ and $\langle V \rangle = 8,650$ km s^{-1} are valid. For $m_c < 15.0$, $\langle m_c \rangle = 16.1$ and $\langle V \rangle = 12,700$ km s^{-1}.

From the differences in $\langle HM \rangle$ one finds that compact galaxies with absorption spectra show a significant redshift anisotropy in the same direction as previously found[1,2]. The anisotropy decreases from the maximum at $14 < m_c \leqslant 15$, especially towards fainter magnitudes. This favours the view that the effect is not due to galactic or intergalactic absorption since absorption would also cause an apparent anisotropy at large distances. This view is supported also by Fig. 1 which gives the distribution of the galaxies on the sky and information on individual HM and shows the regions of exceptionally high and exceptionally low absorption as given by Holmberg[4]. Small HM are not associated with areas of high absorption and large HM with areas of low absorption, as would be expected for an apparent anisotropy attributable to absorbing clouds[5].

T. JAAKKOLA*

Observatory and Astrophysics Laboratory,
00130 Helsinki 13, Finland

H. KAROJI
M. MOLES
J. P. VIGIER

Laboratoire de Physique Théorique,
Institut Henri Poincaré, Paris, France

1 Rubin, V. C., Ford, W K. Jr, and Rubin, J. S., *Astrophys. J. Lett.*, **183**, L111 (1973).
2 Le Denmat, G. and Vigier, J. P., *C. r. hebd. Seanc. Acad. Sci. Paris* (in the press).
3 Zwicky, F., *Catalogue of selected Compact Galaxies and of Post-Eruptive Galaxies* (Offsettdruck, Zurich, 1972).
4 Holmberg, E., *Astr. Astrophys.*, **35**, 121 (1974).
5 Hartwick, F. D. A., *Astrophys. J. Lett.*, **195**, L7 (1975).

Pulsar glitches and restlessness as a hard superfluidity phenomenon

SEVERAL pulsars[1] have "restless" behaviour of the period similar to that of the Crab and Vela pulsars[2] which has been explained[3] as due to "microquakes" in the crust. A glitch has also been observed[1] with, possibly, a new type of signature, in PSR1508+55. We suggest that these phenomena can be explained at least equally well by noisiness in the rate of creep of vorticity through the neutron superfluid in the crust, in almost precise analogy to the well known noisy behaviour of flux creep in hard superconducting magnets[4]. In addition, even the macroglitches, especially those in the Vela pulsar, may be caused by the catastrophic release of pinned vorticity.

The lower crust of the neutron star contains fat nuclei bathed in a neutron Fermi gas which is almost surely superfluid[5]. The discussions we have found of this superfluid do not observe that its T_c and energy gap will at most densities be determined by the proximity effect from those of the neutron superfluid inside the nuclei, since the outer Fermi gas is from 1/2 to 1/10 as dense and will probably have a smaller intrinsic gap (see Fig. 1 and ref. 6). The neutrons inside the nuclei will contribute to some extent to the neutron ρ_s, since their phases are coupled by Josephson tunnelling through the surrounding gas even if that gas is not intrinsically superfluid. T_cs and Δs in the 1/2 to 2 MeV range are reasonable, as is a $\rho_s \sim 10^{12}$–10^{14} g cm^{-3}. Because of the tunnelling effect the correlation length will usually be essentially the internuclear distance: the substance is a very hard Type II superfluid[4].

As Pines pointed out, pinning of the quantised vortex lines which are implied by the rotation of the superfluid,

$$n_{\text{vertices}} = 2m\Omega/h \sim 10^{4-5} \, cm^{-2} \qquad (1)$$

can occur in the crust; they will to some extent be pinned by the regular lattice, since the coherence length is never much greater than the internuclear distance, and even more so by cracks, grain boundaries and other features such as are surely created by quakes. We visualise that vortex lines can slide along lattice planes relatively easily, and are blocked especially severely at these features. Experience with pinning in the analogous hard superconductors suggests that the breaking strength of this vorticity pinning may be surprisingly high. To estimate the pinning strength we estimate the energy of a vortex core as

$$E_{\text{core}}/\text{length} = (\Delta^2/E_F)(k_F^3 \xi^2)$$

and the force per unit length exertable by pinning is this

divided by the distance over which the force acts,

$$F/\text{length} = (\Delta^2/E_F)k_F^3\xi \times p \approx k_F^2\Delta p \simeq 10^{20}p \text{ dyne cm}^{-1} \quad (2)$$

where p is a "pinning probability" factor < 1 describing the fractional density and effectiveness of pinning centres (comparable to the factor p in refs 7 and 8). This force is also of the order one would estimate for the strength of the lattice itself, so that vorticity may fracture the structure rather than
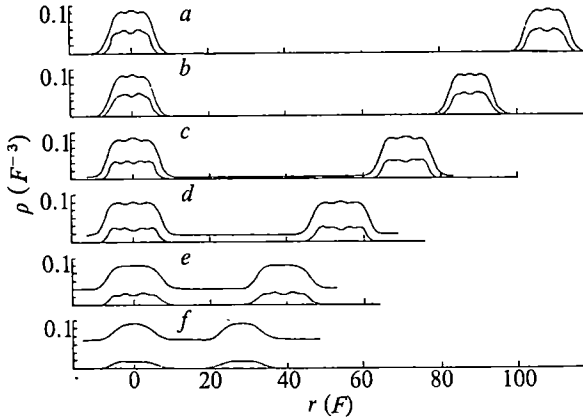
Fig. 1 Proton and neutron density distributions occurring along an axis joining the centres of two unit cells. a, $^{180}Zr_{40}$ with $n_b = 2.79 \times 10^{35}$; b, $^{320}Zr_{40}$ with $n_b = 8.79 \times 10^{35}$; c, $^{1100}Sn_{50}$ with $n_b = 5.77 \times 10^{36}$; d, $^{1800}Sn_{50}$ with $n_b = 2.04 \times 10^{37}$; e, $^{1500}Zr_{40}$ with $n_b = 4.75 \times 10^{37}$; f, $^{982}Ge_{32}$ with $n_b = 7.89 \times 10^{37}$.

unpin from the lattice. Equation (2) must be equated with the Bernoulli force on a vortex:

$$F = (h/2m)\rho v \text{ per unit length} \quad (3)$$

to obtain a critical relative velocity of crust and neutron superfluid at a given point

$$v_c \simeq (2m/h\rho)k_F^2\Delta p$$
$$\simeq 10^8 p \text{ cm s}^{-1}$$

If the difference in angular frequency $\Omega_c - \Omega_n$ is $\Delta\Omega$,

$$(\Delta\Omega)_{crit} = v_c/R \simeq 50p \text{ s}^{-1} \quad (4)$$

Thus even if p is very small there can be strong pinning forces. If it is the actual lattice which pins, p will not be particularly small, $\sim 1/10$–$1/100$ or so. But equation (4) indicates such large values of $(\Delta\Omega)_{crit}$ that we must expect that in most stars the superfluid in the crust is effectively pinned and does not change its Ω, and that in others it will be pinned in some regions and at some times. The former are probably the majority of pulsars which show quiet behaviour[1].

Here we give some suggestions, necessarily rather speculative, as to the actual physics of superfluidity in the pulsar. In the first place, the frictional force suggested by Baym and Pines due to electron scattering on vortex cores is far too small to unpin the vortices; that force may be estimated to be

$$F_{friction} = \rho R\Delta\omega/\tau \text{ per unit volume}$$

$$\lesssim 10^5 \text{ dyne cm}^{-3}$$

where τ is the spin-up decay time of order months introduced by Baym *et al.* If the Bernoulli force due to relative motion of

crust and superfluid is not larger than indicated by equation (2) the vortices stay at fixed positions in the crust rather than flowing with the superfluid as they do in a normal soft superfluid. The Bernoulli force is strong enough that the vortex lines in the core fluid will probably not move with the crust, but will flow with the core superfluid. This must lead to complicated dynamics at the interface; our suspicion is that a turbulent boundary layer will form which breaks up all phase coherence at the crust–core boundary. The theory of these phenomena has been discussed by Greenstein[9]. As he points out, the viscosity due to the turbulence can be of the same order as that due to electron scattering and can be the cause of spin-up. The turbulence and pinning seem likely to damp out any Tkachenko waves or similar collective modes of the vortex lattice. In any case, vorticity need not be perfectly conserved in passing from core to crust, as one might have thought at first.

Thus the dynamics of the core and of spin-ups is little affected. But what does appear here is a second, decoupled, superfluid which in the case of light stars may be a fair fraction $(10^{-2}$–$10^{-1})$ of the neutron superfluid. This crustal superfluid may lag behind the slowing angular velocity of the crust by some cycles per second. The slowing of this superfluid must necessarily take place by the very noisy process of vorticity creep, with concomitant "vorticity jumps" which occur when a particular group of pinning centres become too weak, and may be thought of as the superfluid vortex lattice's equivalent of crustquakes. The reason why these processes will be noisy is that once vortex lines start to move, there is no adequate source of damping to soak up the rotational kinetic energy which is thereby deposited in the vortex lattice. Whether the vortex lattice or the crust itself fractures is not relevant; in either case the event must continue until the stress is relieved.

A second, somewhat independent consequence of superfluid dynamics in neutron stars is the fact that even in the "canonical" view the superfluid never rotates with the same angular velocity as the pulses or the crust, but lags behind by a relative amount τ/T (T is the "lifetime" parameter of ref. 1). The relative lag of crustal superfluid is much greater. It is not obvious that in all cases these angular velocities should be coaxial; this should only be the case if the forces are always simply drag forces and have no couple perpendicular to the axis. In an accreting star such as Her X-1 the accreting matter can exert non-axial couples, and one would not expect Ω_c and Ω_n to be parallel. This can lead to further complications in the dynamics of such systems.

One may estimate that the amount of energy stored in the vortex cores is only $\lesssim 10^{-12}$ of the total rotational energy of the neutron star: using the estimate already given one cannot assume much more than 10^{30} erg in a shell 1 km deep at a radius of 10 km. Nonetheless the associated events may, just as in a superconducting magnet, be much larger than the energy involved in pinning. It is the stored relative rotational energy which is relevant, which can easily be 10^{41} erg. This can be larger than the elastic energy which is responsible for quakes, while the magnitude of strength parameters is similar; thus these events will tend to be larger and more frequent, at least in light stars, than quakes. Thus any of the observed events seem explicable on this basis. We do, however, consider it likely that at least crust quakes will occur as well, and that sometimes the two can occur together.

Superfluidity and hydrodynamic instability mechanisms previously suggested for glitches[9,10] are quite distinct from the present one, and, we believe, less likely. A matter of some importance is that the suggestions for laboratory simulation of the rotating superfluid star become even more meaningful, but that the correct model should have a porous outer layer: the model should be a Reppy superfluid gyroscope[11] with a hole in the centre, not simply a ball of superfluid. Such experiments could help with the vexing question of the boundary layer viscosity and the continuity of vortex lines from core to crust. It would be interesting to study vorticity jumps in such a structure as well as precessional motions.

One of us (P.W.A.) thanks the Technion in Haifa for hospitality and M. A. Alpar for discussions.

P. W. ANDERSON*
N. ITOH

Cavendish Laboratory,
Cambridge CB3 0HE, UK

1 Manchester, R. N., and Taylor, J. H., *Astrophys. J. Lett.*, **191**, L63 (1974).
2 Boynton, P. E., *et al.*, *Astrophys. J.*, **175**, 217 (1972).
3 Pines, D., and Shaham, J., *Nature phys. Sci.*, **55**, 43 (1972).
4 Wischmeyer, C., reported by Kim, Y. B., in *Physics Today*, **17**, 21 (September, 1964).
5 Pines, D., in *Proc. XII int. Conf. Low Temp. Phys.*, (edit. by Konda, E.) (Keigaku, Tokyo, 1971).
6 Negele, J. W., in *Proc. IAU Symp. No. 53* (edit. by Hansen, C. J.) (Reidel, Dordrecht, 1974).
7 Anderson, P. W., *Phys. Rev. Lett.*, **9**, 309 (1962).
8 Kim, Y. B., Hempstead, A. F., and Strnad, R., *Phys. Rev. Lett.*, **9**, 306 (1962).
9 Greenstein, G., *Nature*, **227**, 791 (1970).
10 Packard, R. E., *Phys. Rev. Lett.*, **28**, 1080 (1972).
11 Reppy, J. D., *Phys. Rev. Lett.*, **14**, 733 (1965).

Giant olivine chondrule as a possible later-stage product in the nebula

ABUNDANCES of trace elements, in particular, of rare-earth elements (REE) in the Allende Chondrite and its inclusions have attracted increasing attention from geochemists[1-4]. Based on the abundance characteristics of major elements and trace elements (including REE), Martin and Mason[3] distinguished four distinct groups among the aggregates and chondrules of the Allende Meteorite. We report here on the presence of an olivine chondrule in Allende with REE abundance features quite different from those of any inclusions previously reported.

By chance, we found an extraordinarily large chondrule in Allende (Fig. 1). It measured 7 mm in diameter and appeared light grey with darker coloration towards the margin. In general, the olivine chondrules in this meteorite are only a few millimetres or less in size: previously Tanaka and Masuda[1] studied Allende olivine chondrules which had diameters of about 3 mm. Chemical analysis and X-ray diffraction indicate that the composition of this giant chondrule is much closer to pure olivine than that of the olivine chondrules analysed before[4]. Chemical

Fig. 1 A giant olivine chondrule in the Allende Chondrite. Scale bar, 1 cm.
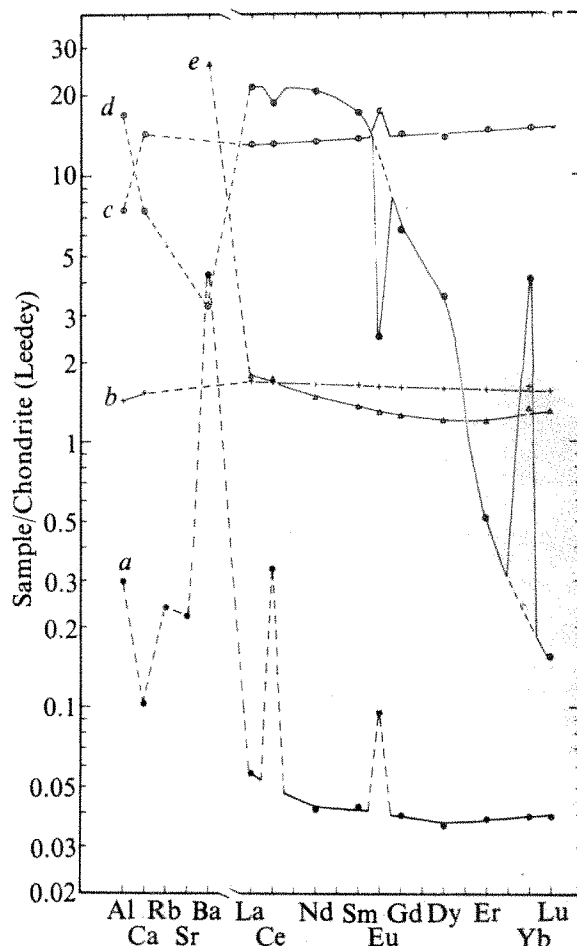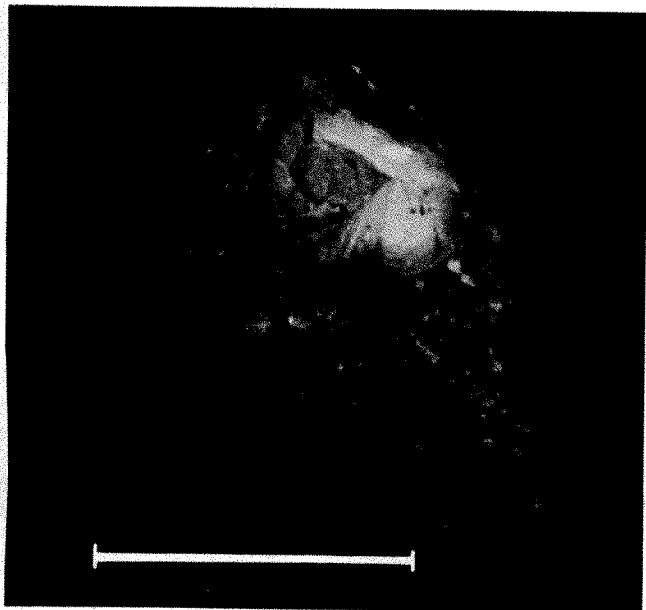
Fig. 2 Chondrite normalised pattern using the Leedey Chondrite as standard. *a*, REE and other elements from the giant olivine chondrule in the Allende Chondrite; *b–e*, other inclusions in the same meteorite; *b*, olivine chondrule; *c*, Ca, Al-rich chondrule; *d*, inclusion G pink; *e*, whole rock.

analyses by Nakamura *et al.* (unpublished) suggest that the material studied here is almost pure olivine (Fo$_{79}$) with perhaps 1.5% feldspathic or glass component.

About 120 mg of the sample were used for determination of REE, Ba, Sr and Rb abundance using a stable isotope dilution technique; Ca and Al abundance were determined by atomic absorption spectroscopy. Analyses are presented in Table 1 and Fig. 2. (For the normalisation of REE abundances, the Leedey chondrite[5] is used as a reference.) The abundances of common REE in the giant olivine chondrule are 40 times lower than those in ordinary olivine chondrules, and there are positive anomalies for Ce and Eu. Although a Yb anomaly is often observed in meteorites[1-3,6], it is not present in this sample. The abundance of Ba is 4 times as high as the reference value, whereas that of Sr is about half the reference value and that of Ca is as low as one-tenth of the corresponding chondritic reference abundance. There is no comparable fractionation among alkaline earth metals in ordinary olivine chondrules[1,3]. The extent of the fractionation of Ba, Sr and Ca may be more or less related to the absolute levels of abundance of common REE and also, presumably, to the size of the olivine chondrule. In addition, a considerable depletion of Al also distinguishes the giant olivine chondrule from ordinary olivine chondrules[1]. In the giant chondrule the contents of Na and K (Nakamura *et al.*, unpublished) are about one-third to one-fifth of those in the Allende bulk sample[2]. Thus, the olivine chondrule studied here is extraordinary not only in size, but also in its chemical features.

It is conceivable that the material constituting the giant olivine chondrule came into being in conditions which favoured

Table 1 Abundances of REE, Ba, Sr, Rb (p.p.m.), Ca and Al (%) in a giant olivine chondrule from the Allende Chondrite

La	0.021	Gd	0.0122	Ba	18.0
Ce	0.32	Dy	0.0140	Sr	2.44
Nd	0.029	Er	0.0098	Rb	0.67
Sm	0.0098	Yb	0.0097	Ca	0.129
Eu	0.0082	Lu	0.00152	Al	0.33

the precipitation of material with an olivine composition only, though these conditions would not have precluded the precipitation of material which gave rise to other chemically different types of chondrules. The very low abundances of common REE imply that these refractory elements had been depleted from the gaseous phase of the nebula by the time that the material represented by this giant olivine chondrule had formed.

The positive Ce and Eu anomalies can be accounted for by the relatively high pressures of the oxides of Ce and Eu[7,8]. Oxides of Ba, Ce, and Eu are more volatile than those of other common REE, and the order of volatility of the metal oxides is Ba > Ce > Eu (ref. 9).

It is worth considering the low content of Rb, one of the comparatively volatile elements. It is possible that although the giant olivine chondrule formed as a later-stage product in the nebula, the temperature at that stage was not low enough to condense the alkali metals in the chondrule.

The characteristics observed in the chondrule are not entirely in line with earlier thermodynamic studies of REE fractionation[10]. (Note the presence of a positive large Ce anomaly, the absence of a positive Yb anomaly, and a mutually unfractionated, flat pattern for common REE from La to Lu.)

In any case, we suggest that the giant olivine chondrule is extremely pure material which could have formed at specific temperature ranges. Broadly speaking, this chondrule of wholly new type may be complementary to Ca, Al-rich aggregate with unfractionated REE (ref. 3) though, in detail, additional components are required.

TSUYOSHI TANAKA

Geological Survey of Japan,
Hisamoto, Kawasaki

NOBORU NAKAMURA
AKIMASA MASUDA

Department of Earth Sciences,
Kobe University, Kobe

NAOKI ONUMA

Department of Chemistry,
University of Tsukuba,
Ibaragi, Japan

1 Tanaka, T., and Masuda, A., *Icarus,* 19, 523-530 (1973).
2 Nakamura, N., *Geochim. cosmochim. Acta,* 38, 757-775 (1974).
3 Martin, P. M., and Mason, B., *Nature,* 249, 333-334 (1974).
4 Wänke, H., Baddenhausen, H., Palme, H., and Spettel, B., *Earth planet. Sci. Lett.,* 23, 1-7 (1974).
5 Masuda, A., Nakamura, N., and Tanaka, T., *Geochim. cosmochim. Acta,* 37, 239-248 (1973).
6 Nakamura, N., and Masuda, A., *Earth planet. Sci. Lett.,* 19, 429-437, (1973).
7 Shchukarev, S. A., and Semenov, G. A., *Dokl. Akad. Nauk SSSR,* 141, 652-654 (1961).
8 Benezech, G., and Foëx, M., *C. r. hebd. Séanc. Acad. Sci, Paris,* 268, 2315-2318 (1969).
9 Samsonov, G. V., in *Physico-Chemical Properties of Oxides* ("Metallurgy" Publishing Office (handbook, Japanese translation) Moscow, 1969).
10 Boynton, W. V., *Geochim. cosmochim. Acta* (in the press).

Heat flow and incipient rifting in the Central African Plateau

EIGHT new heat flow measurements from Precambrian sites in the Republic of Zambia (Fig. 1) range between 55 and 76 mW m^{-2}. Compared with the mean for Precambrian provinces elsewhere, these results are anomalously high by some 50%. This heat flow anomaly persists after taking into account radioactive heat generation of near surface rocks,

and therefore may be interpreted as indicating the presence of anomalously warm material only a few tens of kilometres beneath the Earth's crust. We believe these observations support the hypothesis of an incipient arm of the East African rift system as proposed by Fairhead and Girdler[1,2] and suggest that it may extend into the central African plateau to at least 16°S.

Temperature surveys were made with thermistor probes in more than thirty boreholes distributed over the eight sites, usually several weeks to several months after drilling had been completed. Depths of measurement range from 160 to 1,200 m. Thermal conductivities of solid rock disks sampled from drill cores at 10 to 20 m intervals were subsequently determined on a conventional divided bar apparatus. Heat flow values were then calculated as the product of the least squares geothermal gradient and the harmonic mean conductivity. Mean heat flow values for the sites are given in Table 1.

The results of radioactive heat production measurements on aggregate samples of core chips from several of the sites are also presented in Table 1. No heat production values were obtained for the Ichimpe and Luanshya sites where the boreholes penetrate mainly sedimentary sequences.

In interpreting continental heat flow results it is important to ascertain the tectonic and thermal history of the region. Apart from limited areas covered by Karroo (Permian–Jurassic) sedimentary and volcanic rocks and Kalahari (Pleistocene–Holocene) sands, most of Zambia consists of pre-Silurian, mainly Precambrian, rocks[3] (Fig. 1). The last tectono-thermal event recorded in Zambia is the Damaran–Katangan (Pan-African) episode, which is represented at the northern heat flow sites by isotopic ages of 520 ± 50 Myr, and at the southern sites by ages of 730 ± 50 Myr, (ref. 3). One site, Mkushi, has been unaffected since the earlier Kibaran orogeny and has been dated[4] at >1,635 Myr.

Figure 2 is a graph of continental heat flow as function of age of most recent tectonic mobilisation, after Polyak and Smirnov[5], who identified in some detail the clear trend of diminishing heat flow with increasing age of tectonic province. Also shown in Fig. 2 is the mean value of 66 mW m^{-2} for the eight Zambian sites, plotted collectively at 700 Myr. The Zambian surface heat flow is anomalously high by about 30 mW m^{-2} compared with Precambrian shields elsewhere.

Large surface heat flow can result merely from enhanced radiogenic heat production in the upper few kilometres of the crust. We have compared our heat production measurements with all available data from other Precambrian shields and conclude that enhanced heat production can account for no more than one half of the 30 mW m^{-2} surface anomaly. We therefore infer a heat flux anomaly of at least 15 mW m^{-2} originating in the lower crust or below. Furthermore, as lower crustal and upper mantle rocks are depleted in heat producing isotopes by a factor of five to ten relative to upper crustal rocks, it is unlikely that the source of the residual heat flow anomaly is radiogenic. A more probable source is an anomalous zone of elevated temperatures at depth resulting from an intrusion or from some thermal process in the asthenosphere.

We consider two interpretive models to explain the observed heat flow anomaly. The first involves the computation of surface heat flow in time following a sudden increase in temperature at a given depth. In the plate tectonic model this may correspond to the lithosphere coming to rest over an asthenospheric hot spot or a convective upwelling. If such an event happened during the Miocene[6], plausible solutions restrict the depth of the disturbance to less than 60 km; at greater depths an unacceptably large temperature perturbation is required to produce the observed surface anomaly. A second model involves a temperature perturbation which is spatially
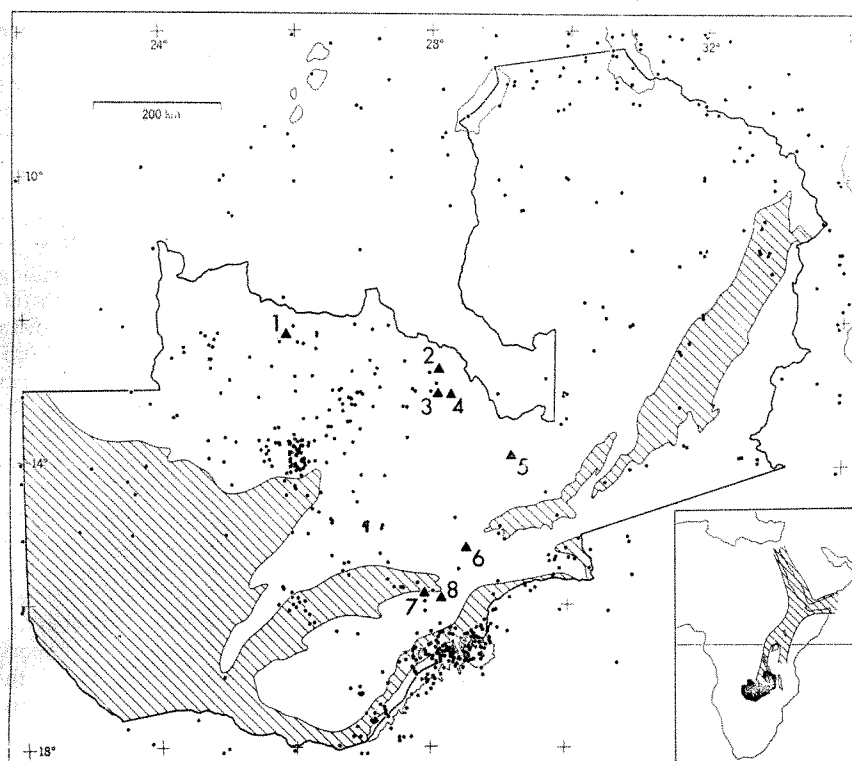
Fig. 1 Location map of the Republic of Zambia showing heat flow sites (▲), earthquake epicentres (•), Precambrian (unshaded) and post Pan-African (shaded) terrains. Epicentres drawn from International Seismological Centre (Edinburgh) Regional Catalogue of Earthquakes, 1965–70. Inset map shows the zone of inferred lithospheric thinning from Fairhead and Girdler[2].

periodic at the base of a moving slab, corresponding to the lithosphere moving over an array of hot and cold spots below. In this model the solution is an upward propagating thermal wave which is both attenuated and phase shifted at the surface. The magnitude of the observed heat flow anomaly places restrictions on both the depth to the temperature perturbations and the velocity at which the plate moves over them. To satisfy the observed surface heat flow anomaly, calculations utilising this model require that the anomalous zone be less than 60 km deep, and also that the lithosphere be moving less than 2 cm yr^{-1} relative to the source pattern.

Arguments can be made for the applicability of either model. It remains unclear whether the central African region of the African plate is at rest in a hot spot frame of reference[6], or is moving slowly northwards[7]. In either case the source of the thermal anomaly cannot lie deeper than 60 km, and if the source is an intrusion from, or the upper boundary of, the asthenosphere, it represents a considerable penetration into or thinning of the continental lithosphere.

Lithospheric thinning has already been proposed by Fairhead and Girdler for the tectonically active East African rift system, to explain slow seismic wave propogation and

high attenuation, and large negative Bouguer gravity anomalies. They map a single zone of thinned lithosphere southward from Ethiopia almost to the Equator where it bifurcates into the eastern and western rifts (Fig. 1, inset). The southernmost part of the western zone departs from Lake Tanganyika, and strikes SSW across the topographically unbroken central African plateau, to approximately 12°S.

How far south does this lithospheric thinning extend? The region of high heat flow in Zambia lies directly on the extension of the western arm of the anomalous zone. We therefore believe the geothermal measurements argue convincingly for an extension of the zone of lithospheric thinning southward through western Zambia, at least to 16°S. Some additional support for this interpretation is drawn from the distribution of seismicity in Zambia (Fig. 1). Two clusters of epicentres are apparent; one in the middle Zambezi Valley, a Mesozoic rift structure which has been seismically active since the impoundment of Lake Kariba, and a second in west-central Zambia. The occurrence of earthquakes in the latter region is curiously uncorrelated with any obvious geological or topographic feature, but does lie nearly wholly within the region of high heat flow and conjectured thin lithosphere. The presence of earthquakes

Table 1 Heat flow and heat production data

	Site	Latitude	Longitude	Heat production* (μW m^{-3})	Heat flow (mW m^{-2})
1	Lumwana	12° 15'S	25° 51'E	2.5	55±8
2	Ichimpe	12° 44'S	28° 07'E		74±4
3	Lumpuma	13° 05'S	28° 03'E	1.8	65±4
4	Luanshya	13° 05'S	28° 19'E		76±4
5	Mkushi	13° 55'S	29° 12'E	1.9	62±2
6	Chalalobuka	15° 13'S	28° 33'E	2.3	66±3
7	Lubombo	15° 49'S	27° 55'E	3.6	71±2
8	Munali Hills	15° 55'S	28° 08'E	1.0	64±4

*Heat production determinations are subject to measurement uncertainties of about 1%. Uncertainties arising from sampling are limited to less than 15% by assembling aggregate samples from throughout each borehole.
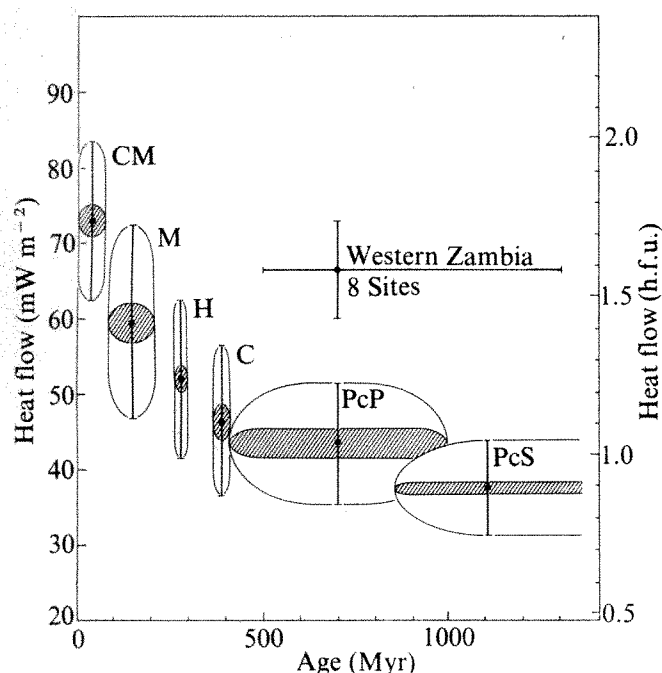
Fig. 2 Mean heat flow from eight Zambian sites superimposed on plot of heat flow against age of tectogenesis after Polyak and Smirnov[5]. Tectonic provinces include: PcS, Precambrian shield; PcP, Precambrian platform; C, Caledonian folding; H, Hercynian folding; M, Mesozoic folding; CM, Cainozoic miogeosyncline. Shaded ellipse represents standard error of the mean heat flow; vertical bar and open ellipse represent standard deviation.

in the anomalous zone suggests that this region, like the mature rifts in East Africa, is dynamic and responding to contemporary tectonic stress.

Further work is required to delineate the boundaries and continuity of this important geophysical anomaly. But our heat flow results to date and the seismicity of western Zambia lend strength to the hypothesis of incipient rifting in the central African plateau.

DAVID S. CHAPMAN
HENRY N. POLLACK

*Department of Geology and Mineralogy,
The University of Michigan,
Ann Arbor, Michigan 48104*

[1] Fairhead, J. D., and Girdler, R. W., *Nature*, **221**, 1018 (1969).
[2] Fairhead, J. D., and Girdler, R. W., *Geophys. J. R. astr. Soc.*, **24**, 271 (1971).
[3] Drysdall, A. R., Johnson, R. L., Moore, T. A., and Thieme, J. G., *Geologie en Mijnbouw*, **51**, 265 (1972).
[4] Snelling, N. J., Hamilton, E. I., Drysdall, A. R., and Stillman, C. J., *Econ. Geol.*, **59**, 961 (1964).
[5] Polyak, B. G., and Smirnov, Ya. B., *Geotectonics*, **4**, 205 (1968).
[6] Burke, K., and Wilson, J. T., *Nature*, **239**, 387 (1972).
[7] Oxburgh, E. R., and Turcotte, D. L., *Earth planet. Sci. Lett.*, **22**, 133 (1974).

Structure of turbulent boundary layers at maximum drag reduction

WE describe here the results of a preliminary study on boundary layers carried out with an aqueous equimolar solution of cetyltrimethylammonium bromide (CTAB) and 1-naphthol at a total concentration of 508 parts per million

(p.p.m.). Dilute, high polymer solutions in pipe flow can produce a drag reduction which is bounded by a limiting asymptote curve[1], and micellar solutions of CTAB-1-naphthol exhibit drag reduction described by the same asymptote[2,3]. Measurements of the velocity profiles of the micellar solution across a pipe diameter show fairly good agreement with the ultimate profile deduced[4] from pipe friction results. This particular drag reducing solution is suitable for experiments in closed loop systems because of its resistance to permanent mechanical degradation.

The measurements reported here were taken at the base of a small flume using the pulsed hydrogen bubble technique for flow visualisation and qualitative measurement. Essentially, the method is that developed by Kline et al.[5,6].

The working section of the flume was 0.28 m wide by 0.20 m deep and was fed from a large stilling chamber, through a contraction, to produce a uniform flow. The circulation was induced by a variable speed centrifugal

Table 1 Summary of near-wall observations

		Water	CTAB-1-naphthol solution	
U_∞	(m s^{-1})	0.365	0.350	0.250
u^*	(m s^{-1})	0.017	0.013	0.0077
v	(m^2 s^{-1})	1.130×10^{-6}	1.280×10^{-6}	1.270×10^{-6}
F	(burst s^{-1} m^{-1})	290	20	9.0
λ	(m)	0.0073	0.037	0.050
λ^+	$(= \lambda u^*/v)$	115	375	300
T_b	$(= 1/F\lambda)$	0.475	1.35	2.22

pump. A boundary-layer trip wire, 1 mm in diameter, was placed across the floor of the channel at the entry end. The stainless steel cathode wire from which the hydrogen bubbles were liberated was 0.05 mm in diameter and was mounted transversely to the flow on a traversing mechanism situated 0.64 m downstream from the boundary-layer trip wire. Flow patterns were recorded on video tape for subsequent analysis.

Mean velocity profiles in dimensionless form are shown in Fig. 1. The wall shear stress, for the micellar solution was determined from the near-wall velocity profile slope. The water test did not provide sufficient data points within the extremely thin viscous sublayer and in that case a modified Clauser procedure was used to determine the wall shear-stress[5]. Experimental results from the micellar

Fig. 1 Boundary layer velocity profiles: $U^+ = u/u^*$; $Y^+ = yu^*/v$ (where u is the mean velocity at distance y from the wall; $u^* = (\tau_w/\rho)^{\frac{1}{2}}$; $\tau_w =$ wall shear stress; $\rho =$ fluid density; $v =$ kinematic viscosity. ●, Water, ($u^* = 0.017$ m s^{-1}); ⊙, CTAB-1-naphthol solution ($u^* = 0.013$ m s^{-1}); ×, CTAB-1-naphthol solution ($u^* = 0.0077$ m s^{-1}). a, $U^+ = 11.7 \ln Y^+ - 17.0$; b, $U^+ = 2.44 \ln Y^+ + 4.9$; c, free-stream values.
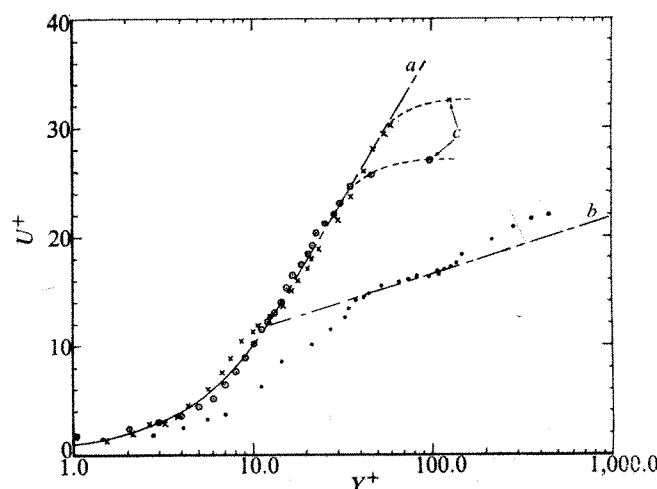
solution seem to be adequately described by Virk's limiting-velocity profile model[4].

The bulk of turbulence production is associated[6] with low speed streaks which originate near the wall in the viscous sublayer and with their subsequent eruption or 'bursting'. From a study of the video records, measurements of the mean transverse spacing of the streaks, λ, the burst frequency per unit span, F, and the time between bursts, T_b, were determined (Table 1). The results obtained with water show fairly good agreement with previous investigations[5-7]. In addition to producing a large friction reduction the micellar solution showed a large increase in the transverse spacing of the streaks and a large reduction in the burst frequency. These changes are more dramatic than those found by previous investigators who used less effective drag reducing solutions[7]. Although the time between bursts T_b, is increased, the value still seems to correlate with the friction velocity, u^*, on the same unique line as that found with water and other polymeric solutions (ref. 7, and B. U. Achia and D. W. Thompson, unpublished).

The shape of the mean velocity profile supports the use of similarity laws for the prediction of boundary-layer skin friction in conditions of maximum drag reduction.

P. S. FREDERICK
A. WHITE

Middlesex Polytechnic,
Engineering, Science and Mathematics,
The Burroughs,
Hendon,
London NW4 4BT, UK

1 Virk, P. S., Trans. Am. Soc. mech. Engrs, 37, ser. E, 488 (1970).
2 White, A., Nature, 214, 585 (1967).
3 White, A., in Viscous Drag Reduction, (edit. by Wells, C. S.), 297 (Plenum, London, 1969).
4 White, A., Nature phys. Sci., 235, 154 (1972).
5 Kline, S. J., et al., J. Fluid Mech., 30, 741 (1967).
6 Kim, H. T., et al., J. Fluid Mech., 50, 133 (1971).
7 Donohue, G. L., et al., J. Fluid Mech., 56, 559 (1972).

Carbon fibre pH-sensitive electrode

VARIOUS forms of carbon have been considered as possible indicator electrodes for use in locating end points in acid-base titrations; these forms have included graphite[1], vitreous or glassy carbon[2] and pyrolytic graphite[3]. Microelectrodes can be used in titrations of small volumes, such as those found in biological cells[4], and certain forms of carbon such as glassy carbon, are exceptionally resistant to oral and tissue fluids[5]. We have examined the electrical potential response of a single carbon fibre to pH changes in aqueous electrolyte solutions.

The potential of a carbon fibre electrode 1 cm long and 7–8 μm in diameter, sealed into a Pyrex glass tube with epoxy resin, was measured against a saturated calomel reference electrode in various buffer solutions in the pH range 1–13, using a Corning EEL 109 digital pH meter used in its millivolt measuring mode. We found a linear relationship between the potential and pH, with a negative slope of about 50 mV per pH unit at room temperature (about 20 °C). The potential in any given buffer, such as 0.05 M potassium hydrogen phthalate, is stable to within 1 mV, within measuring times of a few minutes. Larger variations in potential (and also the slope factor) were, however, observed when the same electrode was examined over a period of weeks; this implies that a carbon-fibre pH electrode of this kind must be calibrated on each day of its use.

The potential of a carbon fibre pH electrode is unaffected by the presence or absence of dissolved oxygen: the same potential was recorded even when pure nitrogen was bubbled through the buffer solution. A small negative shift in potential was, however, observed with temperature increase (about 0.6 mV °C^{-1}). The potential of a carbon fibre electrode is affected by the presence in solutions of strong oxidants such as bromine and cerium(IV) and also by the presence of reductants, such as arsenic(III).

We believe that the observed pH response of the carbon fibre electrode may be caused by the possible ionisation of carboxylic acid groups formed on the surface of carbon by surface reactions with atmospheric oxygen[6,7].

V. J. JENNINGS
P. PEARSON

Department of Chemistry and Metallurgy,
Lanchester Polytechnic, Coventry CV1 5FB, UK

1 Berzik, J., Chemické zvesti., 14, 372 (1960).
2 Dolezal, J., and Stulik, K., J. electroanal. Chem., 17, 1866 (1968).
3 Miller, F. J., Analyt. Chem., 35, 929 (1963).
4 Donald Partridge, L., and Thomas, R. C., Nature, 249, 578 (1974).
5 Von Fraunhofer, J. A., L'Estrange, P. R., and Mack, A. O., Biomed., Eng., 6, 114 (1971).
6 Donnet, J. B., Carbon, 6, 161 (1968).
7 Boehm, H. P., Diehl, E., and Heck, W., Revue gén. Caoutch., 41, 461 (1964).

Electrostatic energy of columbite/ixiolite

A KNOWLEDGE of the crystal structure of an inorganic compound and the appropriate ionic charges is usually sufficient to allow the calculation of the electrostatic energy[1-3]. A more difficult situation is found in crystals where some or all of the cations are disordered so that on the average a particular cation site is occupied by two or more cations with different charges. Very accurate crystal structure refinements, often including refinement of site occupancies, can give a good description of the nature of the disorder and the average composition of each cation site. The electrostatic energy can then be calculated using the average ionic charges on each of the disordered sites. Such an approach has been used to compare the stabilities of ordered and disordered structures of pyrrhotite[4,5] and some complex niobium and tantalum oxides[6]. There seems no theoretical justification for the use of average cation charges for the calculation of the potential energy of a disordered crystal so such calculations must be treated with caution.

Consider a hypothetical crystal structure in which two different cations with different ionic charges occupy four cation sites such that, in the completely ordered arrangement, three are occupied by one ion (A) and the other site contains the second type of ion (B). The ordered arrangement then may be represented by the symbols AAAB. A completely disordered version of this structure determined from a diffraction experiment would be (as indicated above) $3/4A + 1/4B$ for each of the four cation sites. But on the level of an individual unit cell, each cation can be either A or B only and not some fictitious average ion. There are four unique arrangements of 3A and 1B over four sites, namely AAAB, AABA, ABAA and BAAA and the electrostatic energy of each of these ordered arrangements may be calculated by standard procedures. As we do not know how to calculate correctly the energy of the disordered structure, we must deal with what is available; the energies of all the possible ordered arrangements of the cations.

The mineral columbite, $MnNb_2O_6$, offers a good example. In the orthorhombic unit cell are 12 cation sites occupied by 8 Nb and 4 Mn. The charges on the sites in the ordered structure (columbite) are either 5 or 2 and in the disordered structure (ixiolite) the average cation charge on all sites is 4. Barker and Graham[6] have calculated the electrostatic energy of columbite and ixiolite and find that the ordered arrangement is very much more stable than the disordered. This is difficult to reconcile
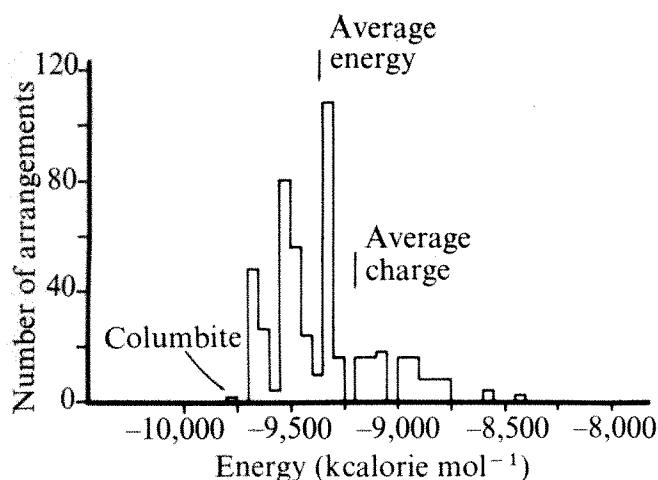
Fig. 1 A histogram of the electrostatic energies of the 495 possible arrangements of 8 Nb and 4 Mn ions in the unit cell of columbite. The "ordered charge" corresponds to the known distribution of the cations, "average charge" represents the energy computed for a structure with average ionic charges (+4) on all cations and "average energy" is the average of all 495 energies.

with the well known tendency of natural columbites to be partially or totally disordered. I suggest that this apparent inconsistency between observation and the calculated energies is due to the incorrectness of the assumption that an electrostatic energy based on average cation charges is an accurate measure of the energy of a real disordered structure.

There are 495 different ways of distributing 8 Nb and 4 Mn over 12 cation sites and the electrostatic energy of each of these structures was calculated along with the energy of the structure containing cations of average charge (+4). The resulting energies are plotted as a histogram (Fig. 1). The calculated energies range from $-9,758$ to $-8,421$ kcalorie mol^{-1} with an average value of $-9,356$ kcalorie mol^{-1} which is greater than the energy, $-9,201$ kcalorie mol^{-1}, for the average charge structure. The largest energy is for the structure of columbite.

Figure 1 shows that many possible arrangements of cations have energies not very different from that for the arrangement in columbite. For example, there are 84 cation arrangements (17%) within 158 kcalorie mol^{-1} of the columbite structure. The spread in energies is probably much less than that shown in Fig. 1 because the geometry of the crystal structure used for the calculations and particularly the sizes of the oxygen polyhedra coordinating the cations are optimum only for the cation distribution in columbite. If the crystal structure of each of the 495 cation arrangements could be optimised, the energy of each would probably increase, the net effect being to compress the histogram towards the position of the columbite structure.

Assuming that the value of $-9,201$ kcalorie mol^{-1} is the true energy of the disordered structure, the question "why does one find disordered crystals in nature when the columbite structure is much more stable?" should be recast in the light of Fig. 1 to "why does one find disordered crystals in nature when hundreds of different ordered arrangements of cations are more stable?" The only reasonable answer is that $-9,201$ kcalorie mol^{-1} is not the correct energy of the disordered structure. Therefore, the method of calculating the energy of the disordered structure is in error and one cannot in general determine whether a particular structure is liable or not to cation disorder by comparing average cation charge energies with ordered charge energies.

I propose that the way to make such a judgment is to calculate the electrostatic energy for all possible combinations of cations as done here for columbite. If there are ordered cation

arrangements with energies close to that of the normal ordered structure, then one would expect that partial or complete disorder of the cations would occur because the existence of other cation distributions with not very different energies indicates that the ions can occupy different sites with relatively little penalty in terms of potential energy. But if there is a large energy gap between the normal structure and other possible ordered distributions, one would not expect to find structures with cation disorder. Figure 1 shows that columbite falls into the first category and so disorder is not surprising. Exactly what energy gap is needed for a structure to have only an ordered arrangement of cations is not known but estimates could be obtained by study of appropriate crystal structures as done here for columbite.

I thank Dr S. Weller for comments. This work was supported by the National Research Council.

R. F. GIESE JR

Department of Geological Sciences,
State University of New York at Buffalo,
4240 Ridge Lea Road, Amherst, New York 14226 and
Wave Propagation Branch, Terrestrial Sciences Laboratory,
Air Force Cambridge Research Laboratories,
Hanscom Air Force Base, Bedford, Massachusetts 01731

1 Ewald, P. P., *Ann. Physik*, **64**, 253 (1921).
2 Bertaut, E. F., *J. Phys. Radium*, **13**, 499 (1952).
3 Tosi, M. P., *Solid State Phys.*, **16**, 1 (1964).
4 Bertaut, E. F., *Acta Cryst.*, **6**, 557 (1953).
5 Tokonami, M., Nishiguchi, K., and Morimoto, N., *Am. Mineral.*, **59**, 1066(1972).
6 Barker, W. W., and Graham, J., *Am. Mineral.*, **59**, 1051 (1974).
7 Wyckoff, R. W. G., *Crystal Structures*, second ed. (Wiley, New York, 1965).
8 Waddesley, A. D., *Revs pure appl. Chem.*, **5**, 165 (1955).

Radiocarbon dates for the Pennine Mesolithic

WE present here a series of ten new radiocarbon dates obtained from charcoal samples collected (1) from sites around Marsden, Yorkshire (Table 1), and formerly stored in the Tolson Memorial Museum, Huddersfield, and (2) from samples collected on upland Pennine sites during the past two years. A single new date from Marsh Benham, Berkshire, is also presented. This short series of dates has some bearing on the relative ages of what Buckley[1] termed the 'broad blade' and 'narrow blade' industries.

The radiocarbon dating of Pennine Mesolithic sites poses several problems. The sizes of the sites vary considerably and there are indications that some may have been occupied for perhaps only a single season, whereas others show signs of lengthy occupation or of having been revisited several times. Datable materials are, however, scarce or absent in all cases, no doubt because of the exposed nature of the upland sites and the constant erosion by high winds and driving rain at these altitudes. The time which elapsed between the abandonment of the sites and their protection by peat formation is unknown, but it was sufficient for most of the datable artefacts to have been destroyed. In the few instances in which charcoal or partially burnt wood has survived in hearths, there is a possibility that contamination has occurred by mineral particles of calcium carbonate and by the down-soaking of humic solutions from the overlying blanket-bog peat. Though these two sources of contamination would have opposite effects on apparent ages they can be eliminated by successive treatments with dilute solutions of hydrochloric acid and alkali. The latter treatment also dissolves a black shiny bituminous substance which sometimes separates from peat and may be confused with charcoal.

Measurements have been made using proportional gas counters with plastic scintillation anticoincidence shielding within a lead castle. Dates have been calculated using the 'Libby half life' of 5,568 yr for the ^{14}C isotope. Where

Table 1 Radiocarbon dates from Mesolithic sites

Site name	Laboratory number	Age (b.p.)	Date (b.c.)	Un-certainty (±)
Lominot III*	Q-1187	9,560	7610	350
Marsh Benham*	Q-1129	9,300	7350	150
Warcock Hill South*	Q-1185	9,210	7260	340
Warcock Hill Site III†	Q-789	8,610	6660	110
Broomhead V†	Q-800	8,570	6620	110
Rishworth Drain†	Q-1166	7,500	5600	210
March Hill II†	Q-1188	6,020	4070	220
March Hill II†	Q-788	5,850	3900	80
Rocher Mill Site II‡	Q-1190	5,830	3880	100
Lominot IV†	Q-1189	5,610	3660	120
Dunford Bridge B‡	Q-799	5,380	3430	80

* Broad blade site.
† March Hill type site.
‡ Rod dominated type site.

samples were small, the technique of dilution with inert carbon dioxide was used. No attempt has been made to calibrate the figures with the bristlecone pine, dendro-chronology curve.

The 'broad blade' industry, now recognised from some 140 locations on the Pennines, was dominated by the manufacture of simple, obliquely blunted points (often retouched on the leading edge) with rare, elongated isosceles triangles and convex-backed blades with a mean width of 8–10 mm. Mellars[2] has pointed out the Maglemosian affinities of this group, comparing the content of these upland sites with Deepcar, Thatcham, Colne Valley[3] and Broxbourne 102 (ref. 4). Confirmation of this purely intuitive correlation is suggested by both computer cluster analysis and a date of 7,615 ± 350b.c. for Lominot site III, "one of two round emplacements"[5] on the eastern side of a hill excavated in 1924 and 1925. That excavation produced 34 microliths[3], end scrapers and awls, 90.9% of the raw material being white flint originating from south-eastern Yorkshire and northern Lincolnshire. Similar material has also been found on other typologically identical sites from both upland and lowland areas[6]. The date agrees, within one standard deviation, with determinations from the Zone V/VI part of the occupation in the swamp-marl at Thatcham Site I/V (ref. 7), (Q-677: 6,830 ± 200b.c.; Q-650: 7,720 ± 160b.c.; and Q-652: 7,550 ± 160b.c.) and with a date of 7,350 ± 150b.c. from a new Maglemosian site, with bone, at Marsh Benham.

The second early site to be dated, Warcock Hill South, differs markedly from the other upland 'broad blade' sites in the shortness and relative width of its microliths, the absence of convex-backed points and points retouched on the leading edge, and in its raw materials. Excavated from "within a space of four square yards" (F. Buckley, private printing, 1924) the industry was based on transparent grey, black and honey coloured flints, identical to those of Star Carr and Flixton Site I (ref. 8) with which industries it bears the closest visual similarity, and with which it links statistically, forming a cluster distinct from that which includes Lominot Site III, Deepcar, Marsh Benham and Thatcham sites I/V–III. Pieces of white flint and chert now housed in the Tolson Museum are clearly of post excavation mixing as both of these raw materials are specifically stated by Buckley (unpublished) to have been absent. The radiocarbon date of 7,260 ± 340b.c. agrees, within the statistical uncertainty, with those from Star Carr (Q-14: 7,607 ± 210b.c.; and C-353: 8,217 ± 560b.c.; and 6,858 ± 490b.c.)[8,10] and could lie, on the basis of the Scaleby Moss diagram, within Zone V, the V/VI boundary here being dated Q-161: 7,059 ± 194b.c. (ref. 10). Warcock Hill South might thus be the contemporary of Flixton Site I, the occupation of which continued into Zone V[11].

Sites of Buckley's 'narrow blade' type break down into three distinct groups, each distinguished both typologically and by the presence and proportions of the raw materials used. The largest group, which we here term the 'March Hill' type industry, is dominated by small scalene triangles, normally less than 5 mm wide, and is known from both upland and lowland locations. These lie on the Pennine and Cleveland moors and also occur in northern Lincolnshire and along the Durham[12,13] and Cumberland[14] coasts. The second group is dominated by straight rod-like microliths blunted along one or two sides. These are known only from high ground on the Pennines and Clevelands. The third group, with many small thomboids, is known in northern England only from two sites, both undated: White Hill North and Red Ratcher on the southern Pennines. The first two groups are confined to northern England, and all three lack the inversely retouched leaf and hollow based (Horsham) points characteristic of the sites in south-eastern England.

Radiocarbon dates for the 'March Hill' type industry cover some three millennia. The earliest, 6,660 ± 110b.c., from Warcock Hill Site III, comes from measurements on charcoal from three 'cooking pits' excavated by Buckley and connected with a circular concentration of chert chippings including only a small number of triangular microliths. The date is identical to one from the Broomhead V Site (6,620 ± 110b.c.)[15,16] and together these represent the earliest known dates for the late Mesolithic type industries in Britain, agreeing closely with the earliest appearance of small scalene triangles in mainland Europe. That the later dates are correct and not too young is hinted at by the presence of a 'March Hill' type industry in terrestrial deposits on top of the early postglacial raised beach at Eskmeals, Cumberland[14], which accumulated, on the evidence from Silverdale Moss, at about 4,640BC (Q-260: 6,590 ± 144b.p.)[17]. Furthermore, the consistency of the two dates from March Hill Site II, measured on charcoal from either the same, or a pair of cooking pits (Buckley's note-books are ambiguous) suggests that the dates are correct.

Some charcoal was excavated from a hearth (Rocher Moss South 2), in an eastwards extension of Rocher Moss South 1 (ref. 18). The site also yielded 35 straight, rod-like microliths, but there were no scalene triangles of the March Hill type. This charcoal gave a date of 3,880 ± 100b.c. (Q-1190), which would, along with a date associated with a similar but smaller industry at Dunford Bridge Site B (3,430 ± 80b.c.; Q-799)[15,16] suggest a date late in the Mesolithic for 'Rod microlithic' industries of this type.

No comment has so far been made of a site at Stump Cross, Grassington[19] where in 1955 24 flint artefacts were found with charred wood, stratified in mud in a rock cleft. Of five pollen samples associated with the flint, four were representative of the classical pollen zone VIIa which reached 30 cm above it and one represented the early part of Zone VIIa and the Boreal–Atlantic, VIIa/VI transition, 5 cm below it. Measurements of the wood yielded a date (Q-141) of 6,500 ± 310b.c. (refs 10 and 20), which is about 1,000 yr earlier than the usually accepted date of this boundary. Possibly, ancient wood had been blown or washed into the pool and became incorporated into the mud. The two rod-like microliths could derive from either a 'March Hill' or 'Rod' type of industry. The mention[19], however, of the occurrence of chert as a raw material, which, in fact, never appears in industries of the 'Rod' type, suggests that the few artefacts recovered belong to the former, rather than the latter, group.

This radiocarbon evidence from the Pennines indicates conclusively that simple 'broad blade' microlithic industries identical to those of Thatcham and Star Carr precede 'narrow blade' (sometimes termed 'Sauveterrian') industries with small scalene triangles and rod-like microliths, which appear just before the middle of the seventh millennium

BC and persist probably until the end of the Mesolithic. Identical successions can be demonstrated on the basis of radiocarbon dating, stratigraphy and pollen analysis, for the valleys of the Lea, Colne and Kennet in south-eastern England, and also for North Wales, whereas the Zone VIIa pollen age for a group of small scalene triangles from Cock Heads, Glaisdale (J. Bartlett, personal communication) contrasts with the Zone IV and V attribution of the Star Carr and Flixton sites in the nearby Vale of Pickering.

We thank Mr J. A. Gilks, of the Tolson Memorial Museum, for access to the Buckley collection and the drawing books in his care, and for allowing samples of charcoal to be removed for radiocarbon dating. Thanks are also due to Dr W. P. Stonehouse, Mr J. Davies, Miss P. Beswick and T. G. Manby.

V. R. SWITSUR
R. M. JACOBI

Radiocarbon Dating Research Laboratory,
University of Cambridge,
5 Salisbury Villas,
Station Road,
Cambridge CB1 2JF, UK

1. Petch, J. A., *Early Man in the District of Huddersfield* (Tolson Memorial Museum Handbook, no. 3, 1924).
2. Radley, J., and Mellars, P. A., *Proc. prehist. Soc.*, 30, 1–24 (1964).
3. Clark, J. G. D., *The Mesolithic Age in Britain* (Cambridge University Press, Cambridge, 1932).
4. Warren, S. H., Clark, J. G. D., Godwin, H., Godwin, M. E., and Macfadyen, W. A., *Jl R. anthrop. Inst.*, 64, 101–128 (1934).
5. Petch, J. A., *ibid.*, 25 (1924).
6. Buckland, P. C., and Dolby, M. J., *Trans. Thoroton Soc.*, 87, 5–33 (1973).
7. Churchill, D. M., *Proc. prehist. Soc.*, 28, 362–370 (1962).
8. Moore, J., *Proc. prehist. Soc.*, 16, 87–100 (1950).
9. Libby, W. F., *Radiocarbon Dating*, 88 (University of Chicago Press, Chicago, 1955).
10. Godwin, H., and Willis, E. H., *Am. J. Sci., Radiocarbon Suppl.*, 1, 63–75 (1959).
11. Godwin, H., in Clark, J. G. D., *Star Carr* (Cambridge University Press, Cambridge, 1954).
12. Coupland, G., *A Mesolithic Industry at 'The Beacons'*, S.E. Durham (Bellows, Gloucester, 1948).
13. Raistrick, A., and Westoll, T. S., *Vasculum*, 19, 139–144 (1933).
14. Cherry, J., *Trans. Cumberland Westmorland antiq. archaeol. Soc.*, 69, 40–53 (1969).
15. Radley, J., Switsur, V. R., and Tallis, J. H., *Proc. prehist. Soc.*, 40, 1–19 (1974).
16. Switsur, V. R., and West, R. G., *Radiocarbon*, 13, 35–51 (1975).
17. Godwin, H., and Willis, E. H., *Radiocarbon*, 3, 60–76 (1961).
18. Stonehouse, W. P., *Bulletin Saddleworth Historical Society*, 2, 36–40 (1956).
19. Walker, D., *Proc. prehist. Soc.*, 22, 23–28 (1956).
20. Godwin, H., and Willis, E. H., *Radiocarbon*, 6, 116–137 (1964).

Possible mechanisms of corona discharge involved in biogenesis

ELECTRIC discharges are very efficient[1] in synthesising amino acids and other organic compounds in conditions which simulate the atmosphere of the primitive Earth. Consequently, they may have been of considerable importance in the origin of life. Corona emission from pointed objects is probably[1] the most effective form of electric discharge involved in prebiotic synthesis. Clearly, coronae occurring in the vicinity of the oceans would be of particular importance, but even though land surfaces generally possess features of sufficient irregularity to permit corona emission in naturally occurring electric fields of moderate strength, it is not obvious that suitable protuberances exist at the ocean surface.

I present here a brief description of experiments which reveal three processes by which coronae can be produced at or near the ocean surface. Two of these processes—the splashing of raindrops in water and the collision of pairs of raindrops—have already been reported[2,3] and are mentioned here only in view of their possible relevance to prebiotic synthesis. The third mechanism is concerned with bubble-bursting.

When a drop falls into water and splashes, a jet of liquid is ejected vertically upwards. It has a diameter close to that of the original drop and may rise several centimetres above the water surface. If such splashing events occur in the presence of an electric field, E, the jet is pulled out further by the field, and when E achieves a critical value, E_c, the tip of the jet becomes pointed and a corona is emitted[2]. The value of E_c decreases as the drop radius, R, increases, and for drops of $R = 3$ mm, about the largest size existing in natural rainfall, $E_c \approx 180$ kV m^{-1}.

When a pair of raindrops collide, the shapes which they assume before separating depend on their sizes, the velocity of impact, and the obliquity of the collision. In some recent experiments[3] a pair of drops, of radii 2.7 mm and 0.65 mm respectively, were brought into collision with a relative velocity of 5.8 m s^{-1} (which is close to the value appropriate to raindrops of this size falling at terminal velocity in the lower atmosphere). The collisions occurred in the presence of a vertical electric field and corona discharges could be detected. When the drops underwent glancing collisions a long filament of liquid was drawn out between them as they separated. In these circumstances, with the field parallel to the filament, conditions were particularly conducive to the production of coronae, and values of $E_c \approx 250$ kV m^{-1} were obtained.

Air forced into the oceans by wave motion or precipitation rises to the surface in the form of bubbles. When a bubble bursts at the surface a jet of liquid rises at high velocity from the bottom of the bubble crater[4] and achieves a height of several millimetres before ejecting a number of drops. It seems possible that the tip of the jet formed when a bubble bursts would provide an effective site for corona emission. Bubbles have been produced by passing air through a tube with a fine capillary tip immersed in water filling a square plastic vessel 0.25 m wide and 0.05 m deep. The bubbles rose to the surface near the centre of the vessel and burst in the presence of a vertical electric field. A reversible polarity, 0–30 kV voltage supply was used to apply a potential difference between the water surface and a smoothed, circular electrode of diameter 0.18 m horizontally mounted centrally above it. The electrode spacing could be varied but was maintained at 0.04 m for the majority of the experiments. Generally, the water was connected to the high voltage supply, the vessel standing on an insulating block. The upper electrode was effectively earthed through an oscilloscope used to monitor the discharges. In other experiments the voltage supply was connected to the upper electrode, or bubbles were allowed to rise about 0.15 m before reaching the water surface. Precautions were taken to ensure that the corona detected was associated with bubble-bursting and not with some spurious process. With the bubble supply switched on, the field was increased slowly until the oscilloscope indicated the occurrence of a corona, which was generally audible. Having thus established a rough value of E_c, observations were made in more detail for values of E around this estimated threshold.

The experiments conducted so far show that corona discharges can occur when bubbles burst at a water surface in fields down to 260 kV m^{-1}. The measured values of E_c are independent of the polarity of the electric field and of the electrode spacing once it is more than about 0.02 m. The independence from polarity is to be expected, in view of earlier experiments[2,5] concerned with coronae resulting from other mechanisms of the disruption of a water surface. A more detailed investigation of the variation of E_c with bubble size, the charges transferred during the emissions and the mechanics of bubble-bursting in electric fields, will follow.

The three processes by which corona discharges may occur in the vicinity of the ocean surface—drop splashing, bubble bursting and raindrop collisions—are all determined by the production of points at a liquid surface and are not significantly affected by the purity of the water or the

presence of trace gases in the air: this has been confirmed experimentally, some measurements having been made using seawater. All these processes require the presence of strong fields associated with thunderclouds. A full investigation of the coronae resulting from raindrop collisions and bubble bursting has yet to be made but it seems likely that the minimum values of E_c for all three processes are in the region of $200 kV m^{-1}$. Such values are known to exist within rainshafts and at the surface of the Earth below thunderstorms. The frequency of occurrence of thunderstorms over the Earth is extremely high—of the order of $100 s^{-1}$. As the most generally accepted mechanisms of thundercloud electrification depend entirely on the basic physical properties of water and ice, it is likely that a similar frequency of occurrence existed in the primitive atmosphere of the Earth. Although present meteorological knowledge does not enable an estimation of the relative importance of the three processes of corona discharge, it seems quite probable that one or more of them could have been primarily responsible for biogenesis.

J. LATHAM

Physics Department,
University of Manchester Institute
    of Science and Technology,
PO Box 88,
Manchester M60 1QD, UK

1 Miller, S. L., and Orgel, L. E., The Origins of Life on the Earth (Prentice-Hall, New Jersey, 1974).
2 Phelps, C. T., Griffiths, R. E., and Vonnegut, B., J. appl. Phys., 44, 3082 (1973).
3 Crabb, J. A., and Latham, J., Q. Jl. R. met. Soc., 100, 191 (1974).
4 Kientzler, C. F., Arons, A. B., Blanchard, D. C., and Woodcock, A. H., Tellus, 6, 1 (1954).
5 Dawson, G. A., J. geophys. Res., 74, 6859 (1969).

Heavy metal release from plants into the atmosphere

INDUSTRIAL activity has been assumed to be largely responsible for the heavy metal content of atmospheric particulates[1]. We have obtained, however, laboratory evidence that higher plants may naturally contribute to the heavy metal composition of the atmosphere. Experiments with radioisotopes have shown that plants are capable of releasing from their foliage a small fraction of the total amount of several elements originally absorbed by their roots. The mechanism giving rise to the release process is as yet not fully understood but seems to be associated with submicron particles. In this way plants could generate local biogeochemical provinces in the atmosphere and reflect underlying geochemical anomalies. Moreover, this

process could also be responsible for the general enrichment of certain heavy metals in the atmosphere relative to the soil and may partly explain the surprising chemical uniformity of atmospheric particulates[1].

The ability of plants to absorb and translocate heavy metals is established[2]. It is also very likely that plants are capable of generating particulate matter in the atmosphere. Went[3] was able to show a very strong correlation between airborne submicron particles and density of vegetation. He suggested that these aerosols were derived from condensation of volatile organic material released from the leaves. On the other hand there is also evidence that fine wax particles can be readily lost from the surface of many plants[4] and that these too could serve as candidates for the formation of particles.

The aim of the experiments reported here was to explore the possibility that heavy metals may be dispersed into the atmosphere by way of a mechanism involving particulate loss from leaves. We report results mainly obtained using radioactive Zn, but several other elements including Pb and Cu have also been studied. Small pea plants (Pisum sativum) were normally used, although broad bean (Vicia faba) and pine tree seedlings (Pinus sylvestrus) have also been used. Plants were grown in liquid culture[5] containing ^{65}Zn (Radiochemical Centre, Amersham, as $ZnCl_2$; specific activity $0.9 mCi mg^{-1}$). When the plants were fully labelled with the radioisotope they were transferred to perspex chambers consisting of foliage and root compartments divided by split diaphragms. The roots were sealed off from the upper chamber using Silastic 738RTV placed around that part of the stem passing through the aperture in the diaphragm. Clean dry air was passed through the chambers at known flow rates and the outgoing air monitored for radioactivity.

Preliminary experiments indicated that radioactive Zn was being released from the plants into the air stream passing through the chamber. Controls were conducted on plants after removing their foliage by cutting at a point on the stem just above the sealed aperture. In these conditions no radioactivity was detected in the outgoing air stream. Studies with a Casella cascade impactor demonstrated that the main Zn-carrying components were associated with submicron particles. For this reason in most experiments the outgoing air was passed through first a Millipore filter ($0.22 \mu m$ pore size) and then a cold finger trap (solid CO_2 and methylated spirit). Radioactivity on the filter and in the frozen water vapour in the cold finger trap (after drying down on planchets) was assayed using a Nuclear Chicago gas flow counter. In most of our experiments the radioactivity given off from the plants was collected in the cold finger condensates and not on the membrane filters. Although the cold finger would tend to trap volatile

Table 1 Release of Zn from leaves of *Pisum sativum*

External concentration (p.p.m.)	Condition	Average Zn level in leaves (p.p.m fresh weight)	Zn release (pg h^{-1} cm^{-2})	Water transpired (ng h^{-1} cm^{-2})	Zn/water ($\times 10^{-12}$)
0.001	Light	7×10^{-4}	0.08	10.0	8.0
	Dark		0.06	6.6	9.1
0.01	Light	5×10^{-2}	0.72	11.6	62
	Dark		0.38	9.0	42
0.1	Light	0.1	4.50	7.0	643
	Dark		2.26	5.6	404
1.0	Light	0.9	21.00	10.6	1,980
	Dark		4.50	8.6	523

Plants were grown in liquid culture medium containing $0.9 mCi$ ^{65}Zn per mg Zn until fully labelled. Illumination was by white light from an incandescent source at an intensity of $100 W m^{-2}$. Temperature $25 \pm 2 °C$. Air in foliage chamber was exchanged every minute. ^{65}Zn released from the plants was detected only in the cold finger trap (see text) and no radioactivity was found in outlet air for the control experiments. Rates of Zn release are expressed in terms of leaf surface area and computed from 4 h sampling times.

organic compounds released from the plant it also acts as a very efficient trap for submicron particles as shown by studies with a condensation nuclei counter. The trapping of particles by this method almost certainly results from diffusiophoresis[6] (the Facy effect[7]) brought about by the development of partial pressure gradients associated with water condensation.

Table 1 gives the results of an experiment with pea plants exposed to external Zn levels in the range 0.001–1.0 p.p.m. In this experiment the amount of Zn released increased with increasing external levels of this element and was enhanced by illumination. In general the clearest evidence of Zn release was usually seen under growing conditions and required the plants to be fully labelled with radiotracer. The quantity of Zn given off, however, was variable and on several occasions only a small amount of the element was released even when the plants were grown at relatively high Zn levels. Although Zn release occurred

agent. Of all the metals we have investigated (Pb, Cu, Hg and Mn) all can be released to varying degrees from the leaves by the above petroleum ether or water treatment.

Evidence that Aitken-sized particles are released from plants, under the experimental conditions used have come from electron microscopy studies. A thermal precipitator with a reciprocating head was connected to the outlet of the chamber and any particles released from the plant which passed through the Millipore filter were deposited on a collector grid which had been covered with formvar film and coated with a layer of carbon. Electron microscopy indicated that needle-like particles up to 2,000 Å long and 300 Å wide had been given off from pea plants.

As yet it is not clear whether these particles are associated with the heavy metal release but they do resemble the wax rodlets which occur on many leaf surfaces including the leaves of peas. The occurrence of these small wax crystalline rodlets on the leaf surface is variable

Table 2 Release of ^{65}Zn from *Vicia faba* growing in unlabelled and labelled solutions after preloading with ^{65}Zn

External Zn concentration (p.p.m.)	Average Zn level in leaves (p.p.m. fresh weight)	Amount of Zn and water released from prelabelled plants in unlabelled solution over 6 h								Amount of Zn and water released from plants when reintroduced to labelled solution	
		Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
		Zn	Water	Zn	Water	Zn	Water	Zn	Water	Zn	Water
1.0	1.45	0.96	13.6	0.40	18.4	0	17.2	0	17.6	4.8	12.0
10.0	22.5	10.8	19.2	7.0	24.4	2.24	27.2	1.72	27.2	20.4	15.6

Samples 1–4 were obtained with plants fully labelled with ^{65}Zn but having their roots in an inactive culture medium. Sample 5 was obtained after reintroducing the roots to a medium containing ^{65}Zn. Daily samples were collected over 6 h so that there was an 18 h gap between sampling. In the case of sample 5 the 18 h period was used to preincubate the plants in radioactive solutions. Sampling was carried out under illumination conditions and in between sampling the plants were exposed to the atmosphere and taken through a day–night cycle. Except for a change in specific activity to 0.09 mCi per mg Zn for the 10 p.p.m. treatment the conditions were the same as for Table 1. Zn and water lost from the plants over the 6 h are expressed in pg cm^{-2} and mg cm^{-2}, respectively.

simultaneously with water loss from the plants there was no obvious quantitative association. There was, however, a requirement that the plants were continuously fed with ^{65}Zn. Table 2 shows that when a fully labelled plant was placed in a culture solution not containing ^{65}Zn, there was a decrease in the ^{65}Zn released even though there would have been little or no change in the ^{65}Zn level in the leaves. Replacement of the plant in the radioactive solution re-established the release process. The release mechanism seems to involve a small Zn pool in the leaf, and the remaining Zn in the leaf is not easily exchanged into this pool. Essentially similar results have been obtained with Cu and Pb but the experiments were more difficult, partly because of variability in the effect and partly because of the relatively low levels of these elements in the leaves. For example, with Pb, levels in the leaves and the outgoing air were reduced by 10 to 50 times of that recorded with Zn when the external levels of these elements were the same (for example, 1 p.p.m.).

The above experiments suggest that the release of ^{65}Zn is associated with submicron particles in the Aitken range. A possible source for these could be small particles of epicuticular wax which occur on the leaf surface and which can easily be removed by certain stress conditions such as leaf expansion[8]. Moorby and Squire[9] tentatively suggested that the loss of these waxes may be responsible for the disappearance of ^{89}Sr from leaves which had been surface-contaminated with radioisotope, an explanation also adopted by others[10]. We have checked to see if the ^{65}Zn absorbed by the roots of our plants is translocated to the vicinity of the leaf surface. Both mild wax extraction procedures[11] and leaching experiments[12] indicate that ^{65}Zn was present near the leaf surface. Approximately 0.22 p.p.m. Zn was detected in a crude wax extraction with petroleum ether from leaves collected from pea plants grown in 1 p.p.m. Zn and under the same conditions about 50 pg Zn per cm^2 leaf surface could be leached with distilled water at pH 6.0, containing 0.1% Tween 20 as a wetting

depending on many factors[4] and could account for the variability observed with the metal release. An alternative explanation for the release could be the production of airborne salt crystals generated by diffusiophoresis associated with rapid transpiration[6]. At this stage we have no positive evidence for either process but the quantitative relationship between heavy metal released and water transpired was highly variable.

Although the rate of metal released is small compared with the total metal content of the leaf it is possible that the release mechanism could give rise to anomalous levels of certain elements in the atmosphere. With a soil water level of Zn at 1 p.p.m. our experiments indicate that the rate of loss of Zn from leaf surfaces can be about 20 pg h^{-1} cm^{-2}. Under these conditions 1 cm^2 leaf surface would contain in the region of 6 ng Zn. Accepting an average leaf area index of 5 (ref. 13) then the rate of Zn release from vegetation to the atmosphere would be 1.0 μg h^{-1} m^{-2}. Such release could, together with human activities, give rise to general heavy metal loadings in the atmosphere. Moreover, our results support those of Curtin *et al.*[14], who reported the release of metals, including Zn, from conifers growing on mineralised soils. Whether the metal-rich particles would normally remain in the Aitken size range in the atmosphere is not yet known but aggregation under the influence of sunlight[3] and cohesion with larger particulates may well occur.

W. BEAUFORD
J. BARBER

Department of Botany,
Imperial College, London SW7, UK

A. R. BARRINGER

Barringer Research Ltd,
Rexdale, Ontario, Canada

1 Pierson, D. H., Cawse, P. A., and Cambray, R. S., *Nature*, 251, 675 (1974).
2 Antonovics, J., Bradshaw, A. D., and Turner, R. G., *Adv. Ecol. Res.*, 7, 1 (1970).

[3] Went, F. W., Slemmons, D. B., and Mozingo, H. N., *Proc. natn. Acad. Sci. U.S.A.*, **58**, 69 (1967).
[4] Martin, J. T., and Juniper, B. E., *Cuticles of Plants* (Arnold, London, 1970).
[5] Hewitt, E. J., *Sand and Water Culture Methods Used in Study of Plant Nutrition* (Commonwealth Bur. Hort. Tech. Commun., 22 East Malling, 1952).
[6] Goldsmith, P., and May, F. G., *Diffusiophoresis and Thermophoresis in Water Vapour Systems in Aerosol Science* (edit. by Davies, C. N.). 163 (Academic, London, 1966).
[7] Facy, L., *Arch. Met. Geophys. Bioklim.*, **A8**, 229 (1955).
[8] Juniper, B. E., *J. Linn. Soc.* (*Bot*), **56**, 413 (1960).
[9] Moorby, J., and Squire, H. M., *Radiat. Bot.*, **3**, 163 (1963).
[10] Chadwick, R. C., and Chamberlain, A. C., *Atmos. Environ.*, **4**, 51 (1970).
[11] Prudy, S. J., and Truter, E. V., *Proc. R. Soc. Lond.*, **B158**, 536 (1963).
[12] Mecklenburg, R. A., Tukey, H. B., and Morgan, J. V., *Pl. Physiol.*, *Lancaster*, **41**, 610 (1966).
[13] Rutter, A. J., in *Water Deficits and Plant Growth*, 2, (edit. by Kozlowski, T. T.) (Academic, London, 1968).
[14] Curtin, G. C., King, H. D., and Mosier, E. L., *J. geochem. Explor.*, 3, 245 (1974).

Conditioned bradycardia in the sea lion *Zalophus californianus*

HEART rate is usually reduced when aquatic mammals dive. The degree of this reduction, known as bradycardia, is remarkable[1-3], and immersion in water has been regarded as important in its initiation[3,4]. The heart of the phocid seal beats slower in forced or restrained dives than during trained or voluntary dives[5,6], and bradycardia occurs during respiratory pauses in air as well as in water[7-9]. Central nervous system (CNS) control of bradycardia has been suggested[5,6,8,9]. We have investigated whether bradycardia can be conditioned in the California sea lion *Zalophus californianus* since cardiovascular responses can be conditioned in man and many other animals[10].

Radiotelemetry devices were surgically implanted into two sea lions under the external thin muscle layer just posterior to the right axilla. One sensing electrode was on the transmitter package with a second on a lead extending across the thorax about 20 cm toward the ventral midline. The telemetry devices contained a magnetic switch by which the transmitter could be turned off between experiments. The transmitters were still in place and functional 18 months after the operations. Frequent physical and haematological examinations revealed no damage to the animals.
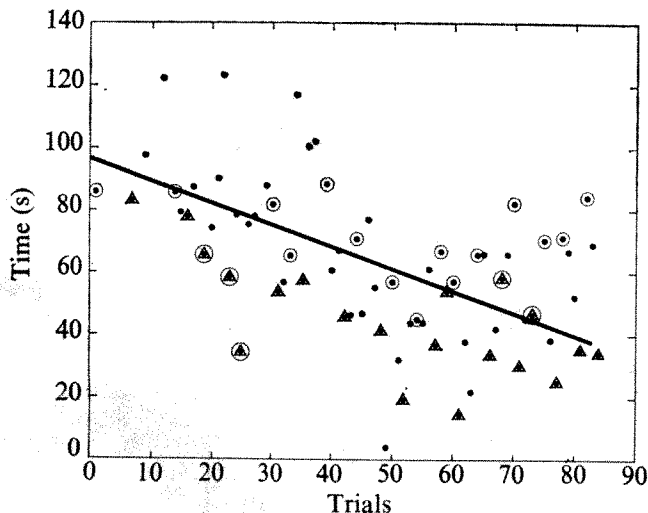
Fig. 1 Each point represents the time required for the sea lion's heart rate to reach 25 beats min⁻¹ (as gauged by the two intervals between three successive QRS complexes). Of the 90 3–5 minute sessions of breath holding, 71 were plotted. Nineteen apnoeic periods were discarded because the heart rate remained above 25 beats min⁻¹, ECG data was incomplete because of radio interference, gross movement of the animal, or other equipment related technical problems. The regression line was fitted to these points: circle with dot, first period of apnoea in each training session; triangle with dot, shortest latency, that is the period of apnoea during each training session when the shortest latency between onset of apnoea and achievement of the 25 beat min⁻¹ rate was achieved; circle with triangle, first and shortest same, that is, the first period of apnoea in a training session also showed the shortest latency.

After a 2 week recovery period after the operation, one sea lion was conditioned to hold its breath in air (that is, the animal's body was out of the water and dry). The trainer would place his hand near the floor and give the command "down". The sea lion would respond by placing its snout against the trainer's hand and holding its breath until a bridging signal (a police whistle) was sounded. In this way 90 sessions of breath holding of 3–5 min duration were recorded from 20 training sessions during about 2 months. In initial trials bradycardia was slow to develop, and about 90 s were required to reach a basal rate of 25–40 beats min⁻¹ from a mean before breath holding of 120 beats min⁻¹. As Fig. 1 shows, the time required to reach a basal rate of 25 beats min⁻¹ became shorter with successive trials.

The other sea lion, which had no previous training, was conditioned to reduce its heart rate in response to an acoustic command signal. Ordinarily a sea lion's heart beat cycles with respiration[7-9], increasing to 100–140 beats min⁻¹ during breaths and decreasing to about 60–90 beats min⁻¹ between breaths. These normal episodes of bradycardia were reinforced during initial training. When a criterion of three successive beats at the slowed rate was reached, a bridging signal was sounded and a reward of fish was given. At first the sea lion was required to reduce the rate to 80 or 90 beats min⁻¹. This requirement was gradually reduced a few beats at a time until a heart rate of 10 beats min⁻¹ was achieved within 20 s after the bradycardia command was sounded.

The sequence of events was as follows: The sea lion entered a cage. The transmitter was turned on by passing a small magnet by the animal's right axilla. The telemetry signal was received on an f.m. radio, decoded and recorded. The audio output of the radio (a 'beep' for each heartbeat) could be heard by the trainer and the animal. The electrocardiogram (ECG) and command signals were recorded continuously on a Grass polygraph (Model 78). The trainer pushed a button to present the bradycardia command signal. When the heart rate reached the previously determined rate within the prescribed time, the trainer sounded the bridging stimulus, then gave the animal a fish through an opening in its cage. Recordings from one such session are shown in Fig. 2a.

To compare conditioned bradycardia with immersion bradycardia, we decided to repeat Elsner's[5] experiments. The same sea lion was then trained, using another acoustic command signal, to immerse its head in a pail of water. The results were very similar to those of Elsner[5]. Immersion was followed by bradycardia (25–40 beats min⁻¹) within a few seconds (Fig. 2b). Immersion bradycardia, however, was not as pronounced as that achieved in the previous heart rate conditioning trials (Fig. 2).

Bradycardia that resulted from simple apnoea was much slower occurring than that resulting from immersion or conditioning. In the case of the first sea lion, which was required to hold its breath for a relatively long period (3–5 min), bradycardia developed more rapidly with succeeding trials. This suggests at least two possibilities. The animal may have learned that more prompt bradycardia made breath holding easier, but learning could not be demonstrated by analysis of data from this experiment. The second possibility is that the sea lion's bradycardia became more prompt as a result of physiological adjustment with repetition (that is, analogous to physical training of an athlete).

The second sea lion definitely learned some response that caused a rapid and profound reduction in heart rate. Certainly, breath holding was involved, but the extent of bradycardia achieved during our conditioning experiments was considerably greater than that during similar periods of apnoea or water immersion. Our findings suggest that the sea lion is capable of some control of its heart rate. Such control can be conditioned directly or may come about

a

Precommand ECG

ECG

Bridge

Bradycardia command

Postcommand ECG

b

Precommand ECG

ECG

Bridge

Immersion command

Postcommand ECG

0 1 2

s
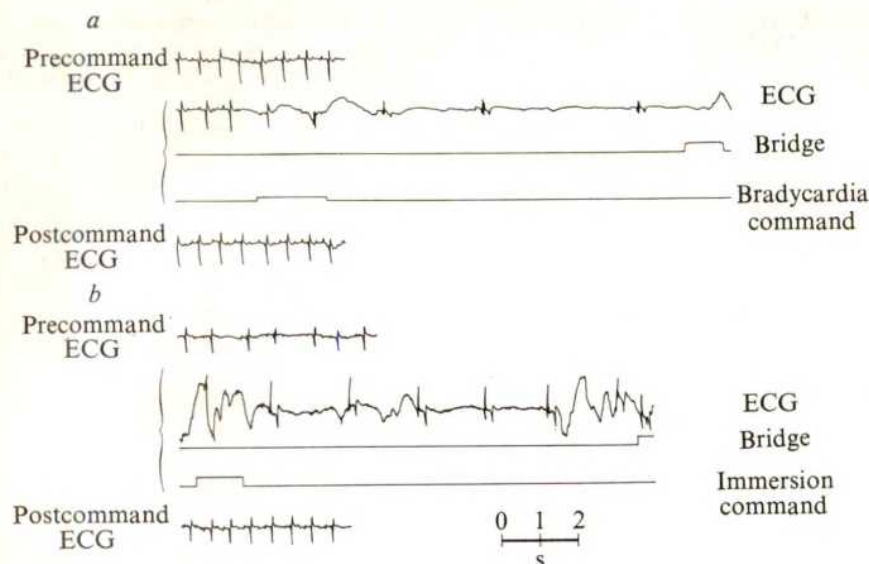
Fig. 2 ECG of the second sea lion. The three different sound stimuli used to signal the animal were indicated on the recordings. *a*, The animal was in a cage in air and dry. The bridge was used to signal correct performance and to indicate to the animal that a fish reward would soon be given. The bradycardia command was meant to signal the animal to reduce its heart rate. *b*, The immersion command signalled the animal to immerse its head in a pail of water. The bridge again signalled correct response after which the sea lion could lift its head from the water and receive a fish.

through some incidental body adjustment of which we are unaware at present (such as, Val Salva's manoeuvre or contraction of respiratory muscles).

The California sea lion has proved to be a tractable laboratory animal that is readily trained to cooperate in experiments. The degree to which it can alter such autonomic processes as bradycardia and peripheral vasoconstriction[1,2,3] may make it an excellent animal for certain studies involving the CNS control of these responses.

We thank R. L. Seeley for electronic support and B. L. Scronce for advice on training the sea lions. Dr W. F. Flanigan and F. G. Wood made helpful suggestions on the manuscript.

SAM H. RIDGWAY
DONALD A. CARDER
WILLIAM CLARK

*Biomedical Division,
Biosystems Research Department,
Naval Undersea Center,
San Diego, California 92132*

1 Scholander, P. F., *Hvalrad. Skr.*, **22**, 1 (1940).
2 Irving, L., *Physiol. Rev.*, **19**, 112 (1939).
3 Elsner, R., Franklin, D., Van Citters, R., and Kenney, D., *Science*, **153**, 941 (1966).
4 Anderson, H. T., *Physiol. Rev.*, **46**, 212 (1966).
5 Elsner, R., *Hvalrad. Skr.*, **48**, 24 (1965).
6 Harrison, R. J., Ridgway, S. H., and Joyce, P. L., *Nature*, **238**, 280 (1971).
7 Hamlin, R., Ridgway, S., and Gilmartin, W., *Am. J. Vet. Res.*, **33**, 867 (1972).
8 Lin, Y., Matsuura, D., and Whittow, G., *Am. J. Physiol.*, **22**, 260 (1972).
9 Jones, D., Fisher, H., McTaggart, S., and West, N., *Can. J. Zool.*, **51**, 671 (1973).
10 Bykov, K. M., *The Cerebral Cortex and the Internal Organs* (Trans. and edit. by Gantt, W. H.) (Chemical Publishing, New York, 1957).
11 Ridgway, S. H., thesis, Cambridge University (1973).

Precocious sexual parasitism in the deep sea ceratioid anglerfish, *Cryptopsaras couesi* Gill

THE eleven families and nearly one hundred species of ceratioid anglerfish are distributed throughout the world's oceans below a depth of 500 m. The Ceratiidae, with two monotypic genera, *Ceratias* Kröyer and *Cryptopsaras* Gill, is one of four ceratioid families whose members exhibit a peculiar and unique mode of reproduction in which dwarfed males become permanently and parasitically attached to the body of a relatively gigantic female. Males of this family have large, forwardly directed eyes, apparently relying entirely on vision for their search and identification of a conspecific female. As in other ceratioid males, they are also equipped with a set of pincher-like denticles at the tips of their jaws for grasping and holding fast to a mate. Attachment is followed by fusion of epidermal tissues, and eventually by a uniting of the circulatory systems, so that the male, whose single function is to produce sperm, becomes dependent on the female for blood-transported nutriment, and the female becomes a kind of self-fertilising hermaphroditic host. Since its discovery 50 years ago, the story of sexual parasitism in ceratioid anglerfish has become a part of everyday scientific knowledge, yet no thoroughly satisfactory analysis of the known facts concerning this remarkable reproductive strategy has been made, in spite of the elegant work of Bertelsen[1]. This report describes sexual parasitism in surprisingly young females of *C. couesi*. Contrary to previous thought, it is now evident that parasitic attachment can take place at an extremely early age immediately following metamorphosis.

It has long been assumed that before acquiring a parasitic male, female ceratioids must mature to an adult stage, that in some forms (especially ceratiids) is of considerable size[2-4]. The smallest known *Ceratias holboelli* with an attached male is 460 mm long[5] (all fish lengths are measured from the snout to the base of the caudal fin). Before this report, the smallest known, sexually parasitised *C. couesi* female was 176 mm long[6]. In recent years, however, two considerably smaller
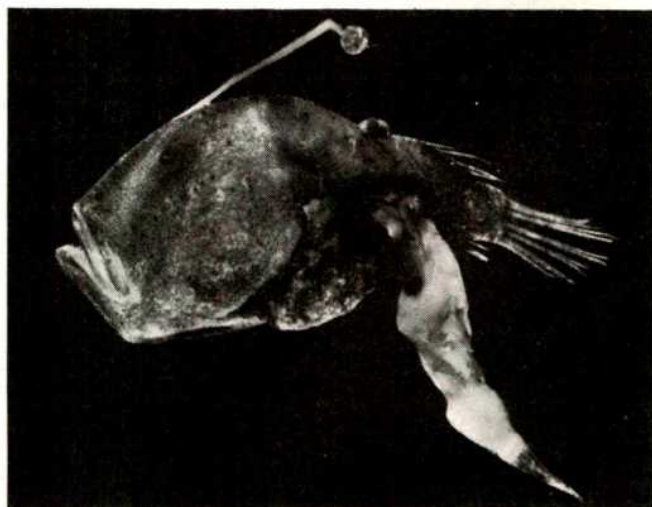
Fig. 1 *C. couesi* female with parasitically attached male, 15.5 and 9.8 mm long (measured from snout to base of caudal fin), respectively (US National Museum, Washington, DC). ×3.75.

C. couesi females with attached males have been discovered. The larger of these is a 77 mm female with a 15 mm male well fused by upper and lower jaws to her belly just to the right of the mid-line (Institute of Oceanographic Sciences, Surrey, UK). Both members of this pair are fully metamorphosed, having darkly pigmented spinulose skin. The testes are large, occupying well over half the volume of the coelomic cavity. In contrast, the paired ovaries are not well-developed, but similar in size to those of non-parasitised females of a similar length.

The second sexually parasitised female *C. couesi* to be described here (Fig. 1; US National Museum, Washington, DC), is remarkable for her small size. At 15.5 mm she is by far the smallest known ceratioid with an attached male. The 9.8 mm male, fused by both upper and lower jaws to the left side of the female just behind the opercular opening, is the smallest known parasitic male of this species. Metamorphosis,

Table 1 Fish lengths and state of gonads for all known parasitically associated females and males of *C. couesi*

Authority	Length*		Gonads	
	Females	Males	Ovaries	Testes
Penrith[12]	356	73.0	?	Fill body cavity
Penrith[12]	322	41.0	?	?
Barbour[13]	290	12.0	Large†	?
Abe and Nakamura[14]	276	12.0	Large†	?
Fast[15]	213	2(27.0–28.0)	Large†	?
Shoemaker[6]	176	3(16.0–37.0)	Large†	?
Unpublished§	173	35.0	Large†	Large‡
Unpublished‖	77.0	15.0	Small	Large‡
Unpublished¶	15.5	9.8	Small	Large‡

*Measured (mm) from snout to base of caudal fin.
†Contain eggs visible to the naked eye, some as large as 0.49 mm in diameter.
‡Length greater than 30% of fish length.
§Los Angeles County Museum of Natural History.
‖Institute of Oceanographic Sciences, Surrey, UK.
¶US National Museum, Washington, DC.

however, is complete in both sexes. The spinulose skin of the female is darkly pigmented; the ovaries are minute. The skin of the male is spinulose, in contrast to free-living males of this genus[7]. Although the testes are considerably larger than those of free-living *C. couesi* males, its small size, lightly pigmented skin, and well-developed eyes, characteristic of free-living males of this species, indicate the youth of this attached male[8]. Bertelsen[9] argued that *C. couesi* females take at least 6 months to reach a length of 15–20 mm, and that males of this species have the shortest free-living stage of any ceratioid examined, probably lasting in most cases less than 6 months. If it can be assumed that the parasitic relationship between these two has not impaired normal growth rates, then the members of this attached pair are both quite young, perhaps 6 months and certainly less than 12 months old. As tissue fusion between the two is extensive, attachment probably occurred some considerable time before capture when both partners were at an earlier stage of development than indicated by their present size and condition.

Members of the family Ceratiidae are among the most commonly collected ceratioids. *C. couesi* is now represented in collections by well over 200 metamorphosed females and approximately 75 free-living males. In all of this material, only nine examples of sexually parasitised females are known to the author (Table 1). Examination of the size distribution of these nine females with attached males throughout the bulk of the known material of *C. couesi* (Fig. 2), shows a statistically significant progression of increase in incidence of parasitism with increase in size (*P* < 0.001, by χ^2). Only 0.7% of the 143 females 75 mm and smaller are parasitised by a male. Of the 158 females 150 mm or less, only 1.3% are parasitised, whereas 50% of the 14 females greater than 150 mm are parasitised.

It seems that a female *C. couesi* is able to elicit a search response in a conspecific male, as well as provide cues for specific identification by that male, at a surprisingly early age. Clearly, however, such early attachment is rare.

Gonadal development and sexual maturity of both males and females seems to be dependent on their mutual presence in an obligatory, sexual parasitic association. At least four of the nine known attached males of *C. couesi* have well-developed testes, yet no free-living male with large testes has ever been discovered. Similarly, gravid females are known, but none without an attached male (Table 1). Of the nine known parasitised females of *C. couesi*, most have ovaries with eggs in various stages of development (Table 1). The two
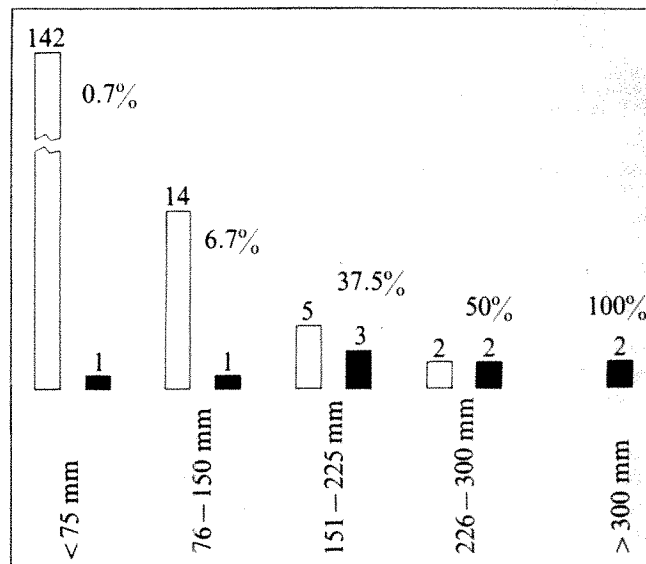
Fig. 2 Distribution by length (measured from snout to base of caudal fin) of nine known sexually parasitised females throughout the bulk of known material of *C. couesi*, showing a statistically significant progression of increase in incidence of parasitism with increase in female size (observed values are significantly different from the expected by χ^2, *P* < 0.001). Material is divided into five size classes of 75 mm each. White bars and black bars indicate number (above column) of non-parasitised and parasitised females, respectively. Percentage of parasitised females is indicated for each size class.

smallest examples, however, are sexually immature, yet their attached males have well developed testes. Had these two small pairs survived the nets of biological oceanographers, the male probably would have remained in a state of sexual readiness for some considerable time until the female reached adult size. On the other hand, perhaps the presence of the male would have led to an early maturation of the female. If so, what have so far been called adolescent females are in actuality 'potential' adults only waiting for the parasitic presence of a male to stimulate ovarian development.

That ceratioid anglerfish, alone among vertebrates, have evolved a reproductive strategy of male dwarfism and obligatory sexual parasitism, can be understood when it is realised that population densities of these organisms are low and mobility restricted by a luring mode of energy capture and by an environment that is vast and productively poor. That parasitic attachment can take place at any time during the apparently long life of a ceratioid[10,11], even at a stage immediately following metamorphosis, and thereby making every meeting of a conspecific male and female a potentially sexual affair, makes this solution to the seemingly difficult problem of reproduction in the deep sea, even more remarkable.

I thank E. Bertelsen, T. R. Roberts, K. F. Liem and P. H. Haas for helpful criticism of the manuscript; M. J. Keene, N. R. Merrett and J. Badcock for specimens; and A. H

Coleman and P. M. Chandoha for photography. This work was supported by a grant from the NSF for studies of western North Atlantic Ceratioidei.

THEODORE W. PIETSCH

Museum of Comparative Zoology,
Harvard University, Cambridge,
Massachusetts 02138

¹ Bertelsen E., *Dana Rep.*, 39, 1–276 (1951).
² Regan, C. T., *Proc. R. Soc.*, B97, 395 (1925).
³ Bertelsen, E., *Dana Rep.*, 39, 247, 250, 257 (1951).
⁴ Fitch, J. E., *Bull. So. Calif. Acad. Sci.*, 72, 164 (1973).
⁵ Abe, T., *Proc. Japan Acad.*, 43, 797–800 (1967).
⁶ Shoemaker, H. H., *Copeia*, 1958, 143–145 (1958).
⁷ Bertelsen, E., *Dana Rep.*, 39, 138 (1951).
⁸ Bertelsen, E., *Dana Rep.*, 39, 144 (1951).
⁹ Bertelsen, E., *Dana Rep.*, 39, 206 (1951).
¹⁰ Fitch, J. E., and Lavenberg, R. J., *Deep-water Teleostean Fishes of California*, 133, 135 (University of California Press, Berkeley and Los Angeles, 1968).
¹¹ Fitch, J. E., *Bull. So. Calif. Acad. Sci.*, 72, 164 (1973).
¹² Penrith, M. J., *J. nat. Hist.*, 1, 185–188 (1967).
¹³ Barbour, T., *Proc. New Engl. Zool. Club.*, 19, 7–14 (1941).
¹⁴ Abe, T., and Nakamura, C., *Jap. J. Ichthy.*, 3, 95–96 (1954).
¹⁵ Fast, T. N., *Copeia*, 1957, 237–240 (1957).

Inversion heterozygosity and the origin of XO daughters of *Bpa*/+ female mice

THERE has long been an interest in developing mouse stocks carrying chromosome inversions for mutation testing. This interest stems from the fact that heterozygosity for a paracentric inversion, that is, a structural rearrangement in which a chromosome segment that does not include the centromere is rotated through 180°, results in suppression of recombination in the inversion region. This is caused by the failure of recovery of the products of single crossovers in this region, because dicentric chromatids and acentric fragments are produced that either fail to give functional meiotic products or result in the formation of unbalanced zygotes which are generally eliminated. Recombination between the pair of chromosomes concerned is, therefore, confined to any crossovers in the non-inverted regions and to the rare two-strand double crossovers which may occur within the inversion region if it is a long one. This property of inversion heterozygotes enables the recovery, in the progeny, of the structurally normal homologue largely intact and any recessive mutation located on it can be readily detected if suitable genetically marked stocks are used. In the simplest case, when the mutation is a recessive lethal and the inversion is located in the X chromosome, detection is either by the absence of half the males, or if the inversion itself is marked by a recessive lethal (as in the C1B method devised by Muller for *Drosophila melanogaster*), by the absence of all the males.

Roderick[1] has made a systematic search for autosomal inversions in the mouse but his method does not recover inversions in the X chromosome. An X chromosome inversion, however, besides being simpler to use, would be far more efficient for mutation testing primarily because detection could be achieved in only two generations rather than the three that would be required using an autosomal inversion. We now report the finding of such an inversion among the descendents of a male that had received a fractionated dose to the spermatogonia, of 12 × 50 rad X rays given at weekly intervals. It was found associated with bare patches (*Bpa*), a mutation which proved to be sex-linked and male lethal[2]. The original *Bpa*/+ females produced, among their progeny, considerable numbers of XO daughters all of the rare OXᴾ type[3]. This enhanced capacity to produce XO progeny was subsequently shown to be separable from *Bpa* and was termed *Fxo* (ref. 4). The presence of *Fxo* also suppressed crossing over between *Bpa* and *Ta* or *Blo* and it was suggested that a structural mutation of the X chromosome, perhaps an inversion, might be involved[4].

A long paracentric inversion of the X chromosome has now been demonstrated cytologically in *Bpa Fxo*/++ animals and shown to be absent from *Bpa*+/++ animals; therefore the symbol *Fxo* has been withdrawn and replaced by In(X)1H (ref. 5) in accordance with the standardised nomenclature for the mouse[6].

The inversion was first identified cytologically by the presence of characteristic bivalents at diakinesis in oocytes from *Bpa* In(X)1H/++ heterozygotes and was confirmed in Giemsa-banded preparations[7] made from bone marrow. Preliminary observations suggest that it extends from a proximal break point lying just within the dark centric band (A1 of Nesbitt and Franke[8]) to a point distal to band E and that it represents about 85% of the physical X chromosome (Fig. 1).

An average of 30 chiasmata per oocyte has been observed
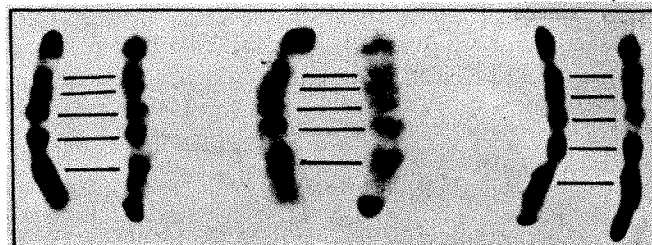
Fig. 1 X chromosomes from three cells heterozygous for the inversion. In(X)1H chromosome is on the right of each pair and inverted in relation to its normal homologue. Lines join presumptively homologous bands. Direct bone marrow preparations stained by trypsin–Giemsa method.

at diakinesis in normal, structurally homozygous mice (C. E. Ford and E. P. E., unpublished). If these are partitioned between bivalents in proportion to the lengths of the chromosomes at somatic mitosis[19], the mean number in the X bivalent would be 1.81. Distributing these in proportion to segment length, the mean number of chiasmata within the inversion region would be approximately 1.54, which would correspond to a genetic length of 77 centiMorgans (cM). This is greater than the total length of the current X chromosome map (75 cM; Fig. 2) and would imply that most, perhaps all, the mapped loci lie within the inversion.
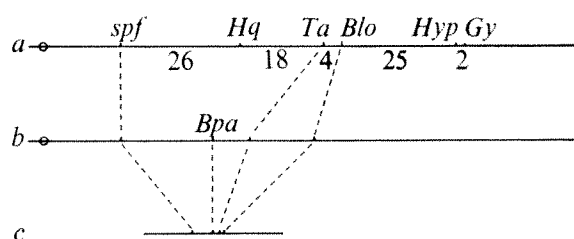
Fig. 2 Map of the X chromosome of the mouse showing the effect on recombination of In(X)1H. *a*, Standard map; *b*, from *Bpa* non-In(X)1H females; *c*, from *Bpa* In(X)1H females. Units represent percentage crossover.

A minimum estimate of the length of the inversion can be obtained independently from the genetic evidence. Data on the recombination between *Bpa* and the other three loci in the absence of In(X)1H (Table 1) indicate that *Bpa* lies between *spf* and *Ta*. Strong crossover suppression had been shown to occur between *Bpa* and *Ta*, and between *Bpa* and *Blo* (ref. 4); it has now also been demonstrated between *Bpa* and *spf* as well (Table 1 and Fig. 2), that is, crossover suppression occurs on both sides of *Bpa*. Also recombination between *Bpa* and either of the markers *Ta* or *spf*, leaves In(X)1H on the unmarked chromosome (Table 1). Together, this evidence implies that *Bpa* lies within the inversion, and therefore to separate *Bpa* from In(X)1H would require a double crossover with one

Table 1 Segregation from: (1) $Bpa+/+Ta \times Blo$ Y*; (2) $Bpa+/+Blo \times Ta$ Y*; and (3) $Bpa+/+spf \times spf$ Y in the presence and absence of In(X)1H

Cross using Bpa In(X)1H chromosome	Female progeny Bpa† OC‡+RC‡	non-Bpa OC‡	RC‡	XO	Male progeny non-Bpa OC‡	RC‡	% Recombination (non-Bpa progeny only)	5% Fiducial limits
(1)	54	131	3‖	50	127	1	1.53	0.42 and 3.91
(2)	77	111	1	67	88	4	2.45	0.79 and 5.72
(3)	22	37§	2‖	23§	39	1	3.80	0.78 and 11.01
Bpa+ Chromosome								s.e.
(1)	66	129	12	—	125	11	8.30	±1.66
(2)	51	55	11	—	58	14	18.16	±3.28
(3)	25	38	15	—	49	12	23.68	±3.98

*Includes data from ref. 4.
†The crossover of any of the genes on to the same chromosome as Bpa is not definitely identifiable, therefore all Bpa offspring are combined.
‡OC, old combinants; RC, recombinants.
§Identified by corneal mitoses. A further 15 spfspf or spf0 have died unclassified.
‖All three recombinants from cross (1) and one from cross (3) have been tested and shown still to carry In(X)1H.

exchange on each side of the Bpa locus. The frequency of separation has been estimated by testing Bpa daughters of Bpa In(X)1H mothers not carrying any other sex-linked gene. Seven of 87 such daughters proved to have lost the inversion ($8.05 \pm 2.92\%$).

If Blo does lie outside the inversion, recombinants between Bpa and Blo in the presence of the inversion would include all Bpa-In(X)1H double crossovers plus any crossovers between the distal break point of the inversion and the locus of Blo. But the estimated recombination between Bpa and Blo (2.45%) is less than between Bpa and the inversion (8.05%); Blo therefore probably lies within the inversion. The same argument can be applied to Bpa and spf (3.80%), and spf more likely than not lies within the inversion. The genetic data are, therefore, consistent with an inversion greater than 48 cM long.

At the stage when the tentative hypothesis for an inversion was first put forward the non-Bpa In(X)1H chromosome had not been obtained[4]. Subsequently both +In(X)1H/++ and +In(X)1H/Y animals have been produced. The females as expected, give a similar percentage of XO progeny to their Bpa In(X)1H/++ counterparts, but the males do not give XO offspring.

A greatly increased frequency of XO daughters would be anticipated among the progeny of females heterozygous for an X chromosome inversion. The dicentric chromatid resulting from a single chiasma within the inversion region would be expected to lead to a chromatid bridge at anaphase I. Whether this was left on the spindle, broken, or incorporated intact into the secondary oocyte (or polar body) nucleus, its eventual elimination would be inevitable, and although the dicentric itself has not yet been recognised a first rough assessment suggests that the frequency of bivalents with a single chiasma in the inverted segment is about 0.4. Elimination could occur either at meiosis or subsequent to fertilisation. In either case an equal frequency of XO and OY individuals would be expected. Crossing over within the inversion is necessarily excluded in In(X)1H/Y males and no XO offspring would be expected.

On the basis of all this evidence, therefore, it seems certain that we are dealing with a very long sex-linked inversion. The occurrence of double crossing over would reduce its efficiency for mutation testing compared with the Muller-5 stock of Drosophila although this may be more than compensated for by the accurate estimates of probability of mutation that would be possible. In any case a technique based on the In(X)1H inversion, already marked by the male-lethal mutation Bpa, would be far more efficient than the methods at present in use for estimating the frequency of sex-linked lethals in mice[10].

E. P. EVANS

MRC External Staff,
Sir William Dunn School of Pathology,
South Parks Road, Oxford, UK

RITA J. S. PHILLIPS

MRC Radiobiology Unit,
Harwell, Didcot,
Oxfordshire OX11 0RD, UK

1 Roderick, T. H., Mutat. Res., 11, 59–69 (1971).
2 Phillips, R. J. S., Hawker, S. G., and Moseley, H. J., Genet. Res., Camb., 22, 91–99 (1973).
3 Russell, L. B., Science, 133, 1795–1803 (1961).
4 Phillips, R. J. S., and Kaufman, M. H., Genet. Res., Camb., 24, 27–41 (1974).
5 Phillips, R. J. S., Mouse News Letter, 52, 35 (1975).
6 Mouse News Letter, 51, 2 (1974).
7 Gallimore, P. H., and Richardson, C. R., Chromosoma, Berl., 41, 259–263 (1973).
8 Nesbitt, M. N., and Francke, U., Chromosoma, Berl., 41, 145–158 (1973).
9 Dev, V. G., Grewal, M. S., Miller, D. A., Kouri, R. E., Hutton, J. J., and Miller, O. J., Cytogenetics, 10, 436–451 (1971).
10 Searle, A. G., Adv. Radiat. Biol., 4, 131–207 (1974).

Limited potential of circulating haemopoietic stem cells

IT has long been known that animals irradiated with a lethal dose may recover if the spleen is shielded during exposure[1], or if normal bone marrow or spleen cells are subsequently injected intravenously[2]. Recovery has been shown to be the result of repopulation of the damaged haemopoietic tissues by stem cells from the donor[3]. It was later found that cells with at least some of the characteristics of stem cells were present in the blood of normal mice[4–6]. These characteristics included the formation of spleen colonies and the ability to promote recovery from lethal irradiation, at least in the short term. Some studies[7] have suggested that circulating stem cells are equivalent to those in bone marrow, while others[8] have indicated certain differences.

In spite of evidence that at least some stem cells are mobile, other data imply that the actual interchange of stem cells between different bone marrow sites may normally be limited or absent[9–14]. Calculations[14] show that, at most, only a small proportion of circulating stem cells enter the bone marrow and find haemopoietic clones. A possible explanation for this is that circulating stem cells have a limited capacity for self renewal. This hypothesis has been tested in two experimental systems.

The first approach was to investigate the relative regeneration of spleen colony-forming units (CFU)—stem cells which home to the spleen—in spleens carrying colonies derived from bone marrow or blood. The experimental scheme and results of four experiments are shown in Table 1. In addition, radioiodinated 5-iodo-2-deoxyuridine (IUdR) was injected into mice in two of the experiments to measure DNA synthesis (Table 2).

Table 1 CFU renewal indices (RI) in the spleens of lethally-irradiated CBA male mice injected with bone marrow (BM) or blood cells*

1 Exp.	Primary recipients		Spleen cells transferred to each secondary recipient			Secondary recipients	
	2 Nucleated cells injected ($\times 10^{-6}$)	3 Mean no. spleen colonies†	4 No. of nucleated cells ($\times 10^{-6}$)	5 No. of colony equivalents	6 Mean no. spleen colonies†	7 CFU RI	8 Ratio of RI —BM: Blood
1 BM	0.1	14.3 (7)	1.4	2.5	21.0 (10)	8.4	15.6
Blood‡	2.2	3.3 (7)	4.6	2.3	1.3 (8)	0.5	
2 BM	0.05	9.6(11)	1.4	1.8	8.0 (8)	4.4	>48.5
Blood	4.0	3.0 (5)	2.4	1.5	0.0 (8)	< 0.1	
3 BM	0.05	14.2 (8)	5.0	4.5	14.3 (6)	3.2	18.3
Blood	4.5	14.9 (7)	13.8	9.0	1.6(12)	0.2	
4 BM	0.05	11.9(12)	4.5	4.8	11.3(20)	2.4	9.5
Blood	9.0	21.2 (5)	11.6	14.8	3.7(20)	0.3	
10-d assay:			1.5	1.6	17.4(19)	11.2	8.6
			16.8	15.9	20.9(20)	1.3	

*Normal BM or blood cells were injected intravenously to lethally-irradiated (900 rad) primary recipients. Eight days later the recipients were killed. Their spleens were divided into two groups; half were fixed for counting of spleen colonies; the remainder were pooled and cell suspensions were prepared in Hanks' solution for intravenous injection to secondary 900 rad-irradiated recipients. These were killed 8 d later, after injection of ^{125}I-IUdR (see Table 2), and their spleen colonies counted. The RI (column 7) was calculated as column 6/column 5 (that is, CFU per colony equivalent injected). Irradiated, non-injected control mice never developed spleen colonies.
†Numbers of mice in parentheses.
‡Heparinised blood was injected whole (experiment 1), or after sedimentation of erythrocytes with Plasmagel (Lab. R. Bellon, Neuilly, France).

The stem cell population derived from blood, assayed as CFU, renewed its numbers far less effectively within 8 d than that derived from bone marrow. The few colonies that did develop in the secondary recipients of blood-derived spleen cells were mostly small, and barely detectable without a hand lens; recipients of marrow-derived spleen cells, on the other hand, developed colonies of normal size. DNA synthesis (and thus cell proliferation) was also relatively slight, although the difference between the groups was less than would have been predicted from the colony counts. Some of the cell proliferation may be attributable to the descendants of injected splenic lymphocytes (originally derived from the primary blood inoculum); terminally differentiating microcolonies of haemopoietic cells may also contribute. The circulating stem cells of mice rendered anaemic with phenylhydrazine have also been shown to have relatively poor powers of self renewal[15].

In experiment 4, the doubling time of CFU between day 8 and day 10 was the same in marrow-derived and blood-derived colonies (20–21 h), which agrees with the data of Gidali *et al.*[8].

The overall regeneration of haemopoietic populations derived from blood and bone marrow stem cells was compared directly by means of chromosome markers. Groups of mice were injected with a mixture of syngeneic bone marrow and blood cells distinguishable by the possession of one or two T6 chromosomes and killed at intervals for cytological examination of mitotic cells[16]. Although the presence of the markers does not affect the proliferative fitness of a population[17], two experimental groups were set up with reciprocally marked donor cells as a precaution. The cell doses were weighted 100-fold in favour of blood, so as to obtain roughly equal numbers of stem cells initially. The suspensions were tested individually for their CFU content. During the second week after injection, the contribution of blood-derived cells to the mitotic populations in the haemopoietic system dropped sharply. By day 23 they constituted less than 5% of the total donor-derived population (Fig. 1).

These experimental results imply that blood and bone marrow stem cells differ markedly in their self renewal, and in the size of descendant populations which they produce in identical conditions. A possible interpretation of the spleen colony data, taken in isolation, would be that blood CFU

Table 2 Splenic uptake of IUdR in lethally-irradiated recipients of cells from colony-containing spleens*

Exp.	Cell injected to primary recipients	Splenic IUdR uptake (geometric mean) in secondary recipients	
		Total specific uptake/spleen†	Specific uptake per colony- equivalent injected (ratio BM: blood)
2	BM	967 (8)	528
	Blood	182 (8)	121
			(4.4)
3	BM	2,905 (6)	653
	Blood	1,445(12)	160
			(4.1)

*Details of mice are given in Table 1. Three hours before death mice were injected intraperitoneally with 5×10^{-8} mol fluorodeoxyuridine followed after 1 h by 1 μCi ^{125}I-IUdR (specific activity, 5 mCi mg^{-1}: Radiochemical Centre, Amersham) as previously described[30]. After washing out of non-DNA-associated radioactivity, spleens were counted in a Packard crystal scintillation counter. ^{125}I-uptake was expressed as a proportion of the injected activity; counts $\times 10^6$ per injected counts.
†Numbers of mice in parentheses. 'Specific' uptake was that attributable to the donor cell population, after subtraction of the mean uptake in irradiated, non-injected controls.
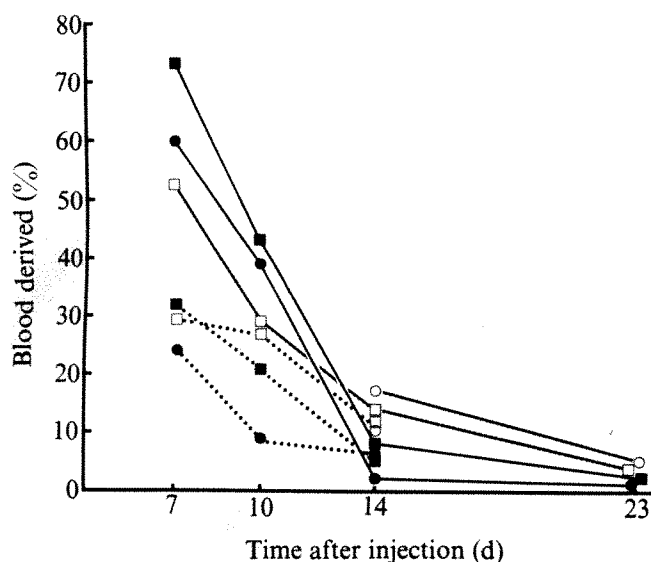
Fig. 1 Identity of mitotic cells in tissues of lethally-irradiated (900 rad) CBA mice injected intravenously with chromosome-marked syngeneic bone marrow and blood cells. Experiment A (- - - - - -): T6 T6 bone marrow (5×10^4 nucleated cells, 4.0 CFU equivalents)+T6/+blood (5×10^6 nucleated cells, 2.5 CFU equivalents) injected. Experiment B (————): T6/+bone marrow (5×10^4 nucleated cells, 2.5 CFU equivalents)+T6 T6 blood (5×10^6 nucleated cells, 8.1 CFU equivalents) injected. Mean number of mitoses scored per tissue: bone marrow 58, spleen 91, thymus 100, lymph nodes 77. ●, Bone marrow; ■, spleen; ○, thymus; □, lymph nodes.

(and/or their CFU descendants) have different homing or migratory properties from marrow CFU, without necessarily differing in their rate of growth. The results of the competition experiment (Fig. 1) argue against such an interpretation. Taken together, the data strongly suggest that although blood CFU are able to engender differentiating clones which grow within 1–2 weeks to perhaps a few million cells, they have, on average, little ability to increase their numbers and thus produce larger and more persistent clones. The presence of a minority of relatively efficient stem cells in the blood is suggested by the similar growth rate of blood-derived and marrow-derived CFU between 8 and 10 d following transplantation.

Calculations based on a stochastic model of haemopoiesis[18] have suggested that in conditions of haemopoietic regeneration in the spleen, the average probability of two new CFU resulting from the division of an existing CFU is 0.62–0.64 (refs 19, 20); in these conditions the CFU pool expands. If this probability is similar for bone marrow stem cells under steady-state conditions, a certain surplus of CFU will be produced continually in the bone marrow. Such a surplus may either be eliminated in situ, or discharged into the bloodstream. These processes may be random, or they may act selectively on, for example, stem cells with a relatively long mitotic history. The present results show that stem cells in blood are not a random sample of those in bone marrow, but are a selected population of generally low proliferative potential. Serial subculture of diploid cells in vitro[21,22] and serial transplantation of tissues[23–25] and haemopoietic cells in vivo[3,20,26–29] cannot be continued indefinitely; after a certain number of divisions the cells cease to multiply. By analogy, it is reasonable to speculate that the CFU normally found in blood are victims of clonal senescence, and have been expelled as waste products from the bone marrow.

Evidence for the low proliferative potential of circulating stem cells is derived almost entirely from work on bachelor male mice of the inbred strain CBA and its congenic line CBA-T6, bred and maintained in conventional conditions. It remains to be seen whether this potential is equally low in other animals and whether it can be enhanced by experimental means.

This work was supported by the Medical Research Council.

H. S. MICKLEM
N. ANDERSON
ELIZABETH ROSS

Department of Zoology,
University of Edinburgh,
West Mains Road,
Edinburgh EH9 3JT, UK

1 Jacobson, L. O., Marks, E. K., Robson, M., Gaston, E., and Zirkle, R. E., J. Lab. clin. Med., 34, 1538 (1949).
2 Lorenz, E., Uphoff, D. E., Reid, T. R., and Shelton, E., J. natn. Cancer Inst., 12, 197 (1951).
3 Micklem, H. S., and Loutit, J. F., Tissue Grafting and Radiation (Academic, New York, 1966).
4 Goodman, J. W., and Hodgson, G. S., Blood, 19, 702 (1962).
5 Micklem, H. S., Transplantation, 4, 732 (1966).
6 Lewis, J. P., Passovoy, M., Freeman, M., and Trobaugh, F. E., J. cell. Physiol., 71, 121 (1968).
7 Kondratenko, N. F., and Chertkov, I. L., Biul. eksp. Biol. Med., 9, 101 (1972).
8 Gidali, J., Feher, I., and Antal, S., Blood, 43, 573 (1974).
9 Ford, C. E., in Ciba Fdn Symp. Thymus, 131 (Churchill, London, 1966).
10 Ford, C. E., Micklem, H. S., Evans, E. P., Gray, J. G., and Ogden, D. A., Ann. N.Y. Acad. Sci., 129, 283 (1966).
11 Carsten, A. L., and Bond, V. P., Expl Hematol., 15, 95 (1968).
12 Maloney, M. A., and Patt, H. M., Science, 165, 71 (1969).
13 Fong, P. L., Maloney, M. A., and Patt, H. M., Blood, 37, 413 (1971).
14 Micklem, H. S., Ogden, D. A., Evans, E. P., Ford, C. E., and Gray, J. G., Cell Tissue Kinet. (in the press).
15 Hodgson, G. S., Cell Tissue Kinet., 6, 199 (1973).
16 Ford, C. E., Appendix in Tissue Grafting and Radiation (edit. by Micklem, H. S., and Loutit, J. F.), 197 (Academic, New York, 1966).
17 Micklem, H. S., Ford, C. E., Evans, E. P., and Ogden, D. A., Cell Tissue Kinet. (in the press).
18 Till, J. E., McCulloch, E. A., and Siminovitch, L., Proc. natn. Acad. Sci. U.S.A., 51, 29 (1964).
19 Vogel, H., Niewisch, H., and Matioli, G., J. cell. Physiol., 72, 221 (1968).
20 Metcalf, D., and Moore, M. A. S., Haemopoietic Cells (North-Holland, Amsterdam, 1971).
21 Hayflick, L., Expl Cell Res., 37, 614 (1965).
22 Martin, G. M., Sprague, C. A., and Epstein, C. J., Lab. Invest., 23, 86 (1970).
23 Krohn, P. L., Proc. R. Soc., B157, 128 (1962).
24 Daniel, C. W., DeOme, K. B., Young, J. T., Blair, P. B., and Faulkin, L. J., Proc. natn. Acad. Sci. U.S.A., 61, 53 (1968).
25 Daniel, C. W., and Young, J. T., Expl Cell Res., 65, 27 (1971).
26 Barnes, D. W. H., Ford, C. E., and Loutit, J. F., Sang, 30, 762 (1959).
27 van Bekkum, D. W., and Weyzen, W. W. H., Pathol. Biol., 9, 888 (1961).
28 Cudkowicz, G., Upton, A. C., Shearer, G. M., and Hughes, W. L., Nature, 201, 165 (1964).
29 Siminovitch, L., Till, J. E., and McCulloch, E. A., J. cell. comp. Physiol., 64, 23 (1964).
30 Pritchard, H., and Micklem, H. S., Clin. exp. Immunol., 10, 151 (1972).

Independent regulation of cellular properties in thermosensitive transformation mutants of mouse fibroblasts

CHEMICAL[1] or viral[2] transformation of fibroblasts in tissue culture results in cells with altered properties (for review see ref. 2). These properties can be broadly subdivided into those associated with changes in the plasma membrane[3] and those associated with intracellular regulatory changes[2]. The former changes include the ability to grow in soft agar[4], the loss of an iodinatable surface glycoprotein[5,6] and an increase in total cell-membrane-associated proteolytic activity[7] on cell transformation. The chief manifestation of intracellular changes is the failure of transformed fibroblasts to cease growing at a specific point (G_1 or G_0) in the cell cycle[8] and thus to express the typical increases in protein synthesis, ribosomal RNA synthesis and DNA synthesis upon growth reinitiation[9]. We have now investigated some of these properties in cell mutants of BALB/c-3T3 which show temperature-dependent changes in many properties characteristic of the transformed state in tissue culture. These mutant cells behave as normal mouse fibroblasts in the regulation of their internal cellular machinery at permissive or non-permissive temperatures but show temperature sensitive changes in those properties we have measured which are considered to be associated with membranes.

Both virus and cell mutants which give rise to temperature sensitive (ts) lesions in many properties characteristic of transformed fibroblasts have been reported[10–12]. Eckhart has

Table 1 Summary of intracellular changes after addition of serum

Cell type	Temperature (°C)	Ratio: newly growing cultures/resting cultures			
		Protein synthesis	rRNA synthesis	DNA synthesis	Cell no.
BALB/c–3T3	32	2.0	2.0	95	1.9
	39	1.8	2.0	86	1.8
ts Cl.1	32	2.0	2.3	35	1.7
	39	2.4	2.0	68	1.9
ts Cl.10	32	2.6	2.3	39	1.7
	39	2.5	1.8	90	2.0
ts Cl.X	32	2.0	1.9	30	1.8
	39	2.0	2.0	55	1.8
Py3T3–Swiss	32	1.1	1.0	1.3	1.1
	39	1.1	1.1	1.4	1.0
SV3T3–BALB/c	32	1.1	1.1	1.0	1.0
	39	1.2	1.0	1.1	1.1

Non-transformed (BALB/c-3T3) mutant (ts Cl.1, ts Cl.10, ts Cl.X) and virally transformed –3T3 (BALB/c–SV3T3, Swiss Py3T3) cells were grown as described previously[13] in Dulbecco's modified Eagles medium (DEM) containing 10% foetal calf serum at either 32 or 39 °C until they formed a confluent monolayer. The medium was removed and replaced with DEM and 1% foetal calf serum for 3 d. Then the medium in half the cultures was replaced with fresh DEM and 20% serum. Protein synthesis was measured by exposing the cultures to 0.2 μCi ml⁻¹ of (^{14}C–U) amino acid mixture (specific activity 57 mCi per matom) from 4 to 6 h at 39 °C or from 8 to 12h at 32 °C; RNA synthesis with 1 μCi ml⁻¹ ^{3}H-uridine at 5×10^{-6} M from 10 until 16 h at 39 °C or from 20 until 32 h at 32 °C. The fraction of cells with ^{3}H-thymidine radioactively labelled nuclei[9] was recorded for cells exposed to ^{3}H-thymidine throughout the DNA synthetic period (from 10 until 30h at 39 °C and from 20 until 60 h at 32 °C), and the cell numbers were recorded after 48 h at 39 °C and 96 h at 32 °C respectively. The radioactivity incorporated into protein was expressed per mg of cell protein. The radioactivity of ^{3}H cytoplasmic ribosomal RNA (rRNA) synthesised in the time period per mg of cell protein was corrected for the differential incorporation rates into the trichloroacetic acid-soluble RNA precursor pool as previously described[9,22]. Previous results for changes in nontransformed fibroblasts[3] cytoplasmic rRNA synthesis by this method agreed with those obtained by other authors from direct measurements of intracellular specific activities of the RNA precursors[22]. Incorporation of ^{14}C amino acids into protein or ^{3}H-uridine into RNA (18S and 28S) increased linearly with the time of exposure to the radioactive macromolecular precursor. Results are expressed as a ratio of the synthesis or cell number after quiescent cultures were induced to grow with fresh serum to those values before the culture medium was changed. Errors in the ratios are approximately 10–15% for protein, RNA synthesis, and cell numbers, and 20–25% for the radioactively labelled nuclei.

isolated different clones of BALB/c–3T3 cells which grow in agar at 32 °C (W.E., unpublished). Cells from these clones show transformed morphology, ability to grow in agar, higher growth rates and higher saturation densities at 32 °C than at 39 °C, although at low concentrations of serum in the tissue culture medium they exhibit density-dependent inhibition of growth at both 32 °C and 39 °C similar to non-transformed cells but unlike the virally transformed cell lines Py3T3 and SV3T3[13]. In this paper three independent ts mutant clones of BALB/c–3T3 cells (ts Cl.1, ts Cl.10 and ts Cl.X) were tested for temperature-dependent changes in various cell properties. Cells were grown in 10% serum and then arrested in their growth by a reduction in the serum concentration to 1%. On addition of fresh medium and 20% serum to the quiescent mutant cells at either 32 or 39 °C they increased the overall rate of protein synthesis, cytoplasmic RNA synthesis (28S and 18S RNA), initiated DNA synthesis and divided (Table 1) just as their parent cell[9]. The time scale for the increase in macromolecular synthesis and cell division was twice as slow at 32 °C than at 39 °C (Table 1). In particular for both the mutant and BALB/c–3T3 cells at 39 °C DNA synthesis was

initiated at 8–10 h, reached a maximum at 18–20 h and was followed by virtually complete cell division by 40 h, whereas for parent and mutant cells at 32 °C DNA synthesis was initiated at 16–18 h, reached a maximum at 34–36 h and was followed by nearly complete cell division by 80 h. Thus the mutant cells were uniquely arrested in growth in the same position of the cell cycle as their parent BALB/c–3T3 cell (G_1 or G_0) at both temperatures and had not ceased growing at random[14]. The fully transformed BALB/c–3T3 cells SV3T3 and Swiss Py3T3 did not show density-dependent inhibition of growth at low serum concentrations, and thus did not appreciably increase rates of macromolecular synthesis and cell division on readdition of 20% serum (Table 1).

Recently the loss of a large, external, transformation-sensitive (LETS) protein has been described after viral or chemical transformation of fibroblasts[5,6]. This has been demonstrated using lactoperoxidase-catalysed iodination. The mutant clones of BALB/c–3T3 cells were arrested in their growth at confluency (Table 1), then radioactively labelled with ^{125}I *in situ*, and the iodination patterns analysed on SDS-polyacrylamide gels (Fig. 1). The radioactive LETS

a b c d e f g h i j
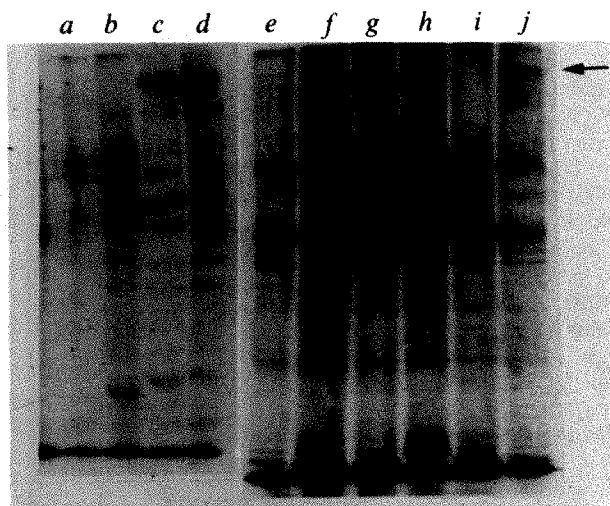
Fig. 1 Autoradiograph of ^{125}I-labelled cells after SDS-polyacrylamide gel electrophoresis. BALB/c–SV3T3 cells grown at: *a*, 32 °C and *b*, 39 °C; BALB/c–3T3 at *c*, 32 °C and *d*, 39 °C; ts Cl.1 at *e*, 32 °C and *f*, 39 °C; ts Cl.10 at *g*, 32 °C and *h*, 39 °C; ts Cl.X at *i*, 32 °C and *j*, 39 °C. Confluent cultures of cells were iodinated as previously described[6] directly on the dish. Before iodination, the cells were washed twice at room temperature with phosphate-buffered saline (PBS), *p*H 7.2 and to each 5-cm dish was added 0.5 ml of PBS containing D-glucose (0.9 mg ml⁻¹) and carrier-free Na^{125}I (400 μCi ml⁻¹). Then 10 μl of an enzyme mixture containing lactoperoxidase (1 mg ml⁻¹) and glucose oxidase (0.1 U ml⁻¹) was added for 10 min at room temperature. The reaction was stopped by the addition of 5 ml of 0.17 M NaI in 0.01 M sodium phosphate buffer, *p*H 7.2. The cell monolayers were then washed twice with PBS, the cells were scraped off the dish with a rubber policeman and rewashed with 0.1 M Tris-HCl (*p*H 6.8) containing 15% glycerol. Samples of 10⁵ c.p.m. were collected by centrifugation, adjusted to 2% sodium dodecyl sulphate and 0.1 M dithiothreitol, and boiled for 2 min. Polyacrylamide gel electrophoresis[23] was performed in a slab gel at an acrylamide monomer concentration of 7.5%. After electrophoresis, the slab gel was dried in a vacuum and autoradiographed for 4 d using Kodak X-ray film RP/R54 to locate the radioactively labelled components.

glycoprotein of nominal molecular weight 250,000 was observed in the parent cells and the ts cells at 39 °C but had virtually disappeared in the ts cells at 32 °C and the BALB/c–SV3T3 transformed fibroblasts. Several other radioactively-labelled components disappeared when the mutant cells were grown at 32 °C instead of at 39 °C, although whether these reflected changes in plasma membrane composition or differential adsorption of serum components to the cells at the two temperatures is unknown. The same pattern was observed if the mutant cells were grown in 10% serum and radioactively labelled when confluent (not shown).

The cell factor that is involved in tumour-associated fibrinolysis occurs in the postnuclear particulate fraction of Rous sarcoma virus transformed chick embryo fibroblasts, although its exact membranous location has not yet been determined[7]. This arginine-specific protease converts serum plasminogen to its enzymatically active form plasmin. The plasmin in turn can be detected by its capacity to hydrolyse ^{125}I-fibrin[15] or the milk protein casein in an agarose overlay assay[16]. Cells were grown as before (Table 1) and assayed for the production of the cell factor by the degree of hydrolysis of casein with either 1% monkey serum, 0.5%

permanently at 32 °C in all the tests used, and *vice versa*. After more than 20 passages in cell culture ts Cl.1 cells started growing in agarose at 39 °C and simultaneously the iodinated LETS glycoprotein was not detectable and the proteolytic activity increased from 0 to +2.

Previous reports have indicated that many of the properties characteristic of the transformed state change in a coordinate manner on viral transformation of fibroblasts. But the isolation of stable SV40-transformed 3T3 cells with partially transformed characteristics[17-19] and SV40-transformed rat embryo cells which continuously express the viral T antigen but coordinately vary in anchorage dependence and plasmin activator production[20], have demonstrated that SV40-induced transformation of fibroblasts can lead to a variety of stable changes. The dependence of BALB/c–3T3 fibroblasts on anchorage for growth may be directly responsible for their failure to induce tumours in animals[21]. We have now shown in the same temperature-sensitive mutant cell that those properties considered to be associated with the surface membrane, the ability to grow in agarose, the disappearance of the iodinatable glycoprotein, the loss of the growth-promoting ability of the fibroblast growth factor[13] and the increase in total cell membrane proteolytic

Table 2 Proteolytic activity and growth in agarose

Cell type	Temperature (°C)	Degree of casein hydrolysis (scale 0 to 4)			Colony formation (%)
		Monkey serum	Dog serum	Plasminogen	
BALB/c–3T3	32	1	0	0	0.3
	39	0	0	0	0.6
ts Cl.1	32	3	3	2	21
	39	0	0	0	0.9
ts Cl.10	32	3	3	2	28
	39	0	0	0	0.5
ts Cl.X	32	4	3	3	25
	39	0	2	0	6.6
Py3T3–Swiss	32	ND	4	3	41
	39	ND	3	3	40
SV3T3–BALB/c	32	4	4	4	ND
	39	4	4	4	ND

For the measurement of fibrinolytic activity the conversion of serum plasminogen to plasmin by the cell activator[7] was measured by the plasmin-induced hydrolysis of casein[16]. Cells grown at their respective temperatures were plated at 10[5] (Monkey serum) or 2 × 10^{5} (dog serum and plasminogen) per 3.5 cm Petri dish in DEM and 10% foetal calf serum. After 3 h the medium was removed, cell monolayers were washed twice with DEM and then 1 ml of a suspension of 0.6% agarose, 2% autoclaved powdered milk (Marvel) mixed with one of 1% African green monkey serum (Flow Laboratories) or 0.5% dog serum (Gibco) or 30 μg ml^{-1} of plasminogen purified from foetal bovine serum (gift of Dr R. Hynes) in DEM was added and allowed to solidify at room temperature. Finally, 1.5 ml of 0.6% agarose in DEM was added, allowed to solidify and the dishes were then incubated at 32 or 39 °C until the casein in the dishes containing SV3T3 cells had completely lysed (approximately 40–50 h at 39 °C and 80–120 h at 32 °C depending on the serum used). The remaining plates were scored on a scale of 0 to +4 (complete hydrolysis) according to the proportion of casein removed. Similar results were obtained with 0.25%, 1% and 2% dog serum, but with 10% dog serum there was virtually no differences for the ts cells at 32 °C or 39 °C. Petri dishes containing no transformed cells but with serum or plasminogen, or containing transformed cells but no serum or plasminogen gave no detectable casein hydrolysis, while 0.005% trypsin (w/v) completely hydrolysed the casein within 48 h at either 32 °C or 39 °C. Fibrinolytic assays[15] using the same cells and sera failed to yield definite differences in the rate of proteolytic hydrolysis of ^{125}I-labelled fibrin (not shown). The ability of different cells to grow in agarose was determined by plating 10[4] or 10[5] cells in liquid medium containing 10% foetal calf serum in 5 cm Petri dishes. Cells were overlayered with a suspension of 0.33% agarose and 10% foetal calf serum in DEM, incubated at 32 or 39 °C, and the colonies counted after 2, 3 and 4 weeks. The percentage (%) of cells which grow in agarose is shown. The figures represent the average of 3 separate experiments (8 Petri dishes per experiment) and 2 microscopic fields of 200 cells were counted per Petri dish. The ts cells formed appreciably smaller colonies at 32 °C than the Py3T3 cells. ND, not determined.

dog serum, or purified bovine plasminogen (Table 2). Calf serum contains an inhibitor of the plasmin assay[15] and thus was not used. The extent of removal of the casein was estimated on a scale of 0 to +4 (complete removal). Thus BALB/c–3T3 and the ts cells at 39 °C showed little or no cell factor activity whereas the ts cells at 32 °C, Swiss Py3T3 and BALB/c–SV3T3 showed considerable activity. Results in Table 2 also confirm that the ts Cl.1 and Cl.10 cells formed 30–50 times more colonies in agarose at 32 °C than at 39 °C, although the differences for ts Cl.X were smaller. Similarly the ts cells show decreased plating efficiencies in agar and in liquid medium at 39 °C than at 32 °C. (W.E. unpublished). In controls (not shown) ts Cl.1 cells grown at 39 °C were grown for several generations at 32 °C and these cells behaved identically to the ts Cl.1 cells grown

activity are coordinately changed with the temperature at which the cells are grown. These mutant cells, however, behave as normal fibroblasts at either temperature with respect to the regulation of their intracellular macromolecular synthesising machinery. Unfortunately it is not possible to test the ability of ts mutant cells to induce tumours *in vivo* at 32 °C and 39 °C.

The coordinate changes observed on transformation may not be reflected in every type of cell. Several non-transformed cells containing LETS protein also show significant levels of fibrinolytic activity whereas some transformed cell lines contain LETS protein but have reduced levels of fibrinolytic activity (E.P., R. O. Hynes, V. Hemmings and L. M. Franks, unpublished). Results with our mutant cells, however, suggest that at least two independent

cell functions control the transition from normal to fully transformed fibroblasts and that in these mutant cells the ability to regulate changes in some cell surface properties is uncoupled from major changes in intracellular events.

P. S. Rudland
E. Pearlstein*
D. Kamely†
M. Nutt

Division of Cell Regulation,
Imperial Cancer Research Fund,
PO Box 123,
Lincoln's Inn Fields,
London WC2A 3PX, UK

W. Eckhart

The Salk Institute, PO Box 1809,
San Diego, California 92112

1 Mondal, S., and Heidelberger, C., *Proc. natn. Acad. Sci. U.S.A.*, **65**, 219–225 (1970).
2 Dulbecco, R., *Science*, **166**, 962–968 (1969).
3 Burger, M., *Biomembranes*, **2**, 247–270 (1971).
4 Macpherson, I. A., in *Tissue Culture Methods and Applications* (edit. by Kruse, P. F., and Patterson, M. K.) 276–283 (Academic, New York, 1973).
5 Hynes, R., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 3170–3174 (1973).
6 Pearlstein, E., and Waterfield, M., *Biochim. biophys. Acta*, **362**, 1–12 (1974).
7 Unkeless, J., Dano, K., Kellerman, G. M., and Reich, E., *J. biol. Chem.*, **249**, 4295–4305 (1974).
8 Pardee, A. B., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1286–1290 (1974).
9 Rudland, P. S., Seifert, W., and Gospodarowicz, D., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 2600–2604 (1974).
10 Dulbecco, R., and Eckhart, W., *Proc. natn. Acad. Sci. U.S.A.*, **67**, 1775–1781 (1970).
11 Renger, H. C., and Basilico, C., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 109–114 (1972).
12 DiMayorca, C., Greenblatt, M., Trauthen, T., Soller, A., and Giodano, R., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 46–49 (1973).
13 Rudland, P. S., Eckhart, W., Gospodarowicz, D., and Seifert, W., *Nature*, **250**, 337–339 (1974).
14 Seifert, W., and Rudland, P. S., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4920–4924 (1974).
15 Unkeless, J. C., *et al.*, *J. exp. Med.*, **137**, 85–113 (1973).
16 Goldberg, A. R., *Cell*, **2**, 95–102 (1974).
17 Smith, H. S., Scher, C. D., and Todaro, G. J., *Virology*, **44**, 359–370 (1971).
18 Scher, C. D., and Nelson-Rees, W. A., *Nature new Biol.*, **233**, 263–265 (1971).
19 Risser, R., and Pollack, R.E., in *Cold Spring Harb. Symposium on Cell Proliferation* (edit. by Clarkson, B., and Baserga, R.), 139–150 (1974).
20 Pollack, R., Risser, R., Conlon, S., and Rifkin, D., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4792–4796 (1974).
21 Boone, C. W., *Science*, **188**, 68–70 (1975).
22 Rudland, P. S., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 750–754 (1974).
23 Laemmli, U. K., *Nature*, **227**, 680–685 (1970).

A "permissive" effect of dexamethasone on the glucagon induction of amino acid transport in cultured hepatocytes

The adrenal glucocorticoids have been shown to be involved in the regulation of cyclic AMP activated metabolic processes such as lipolysis[1], glycogenolysis[2] and gluconeogenesis[3]. The role of the glucocorticoids in the regulation of these processes has been termed "permissive"[4] since it enables the maintenance of the normal responsiveness of cells to hormones which act through cyclic AMP. Glucagon is known to activate the gluconeogenic pathway of mammalian liver at several sites[5] including amino acid transport[6]. We have studied the hormonal regulation of amino acid transport in rat liver parenchymal cells maintained in culture and report here that the synthetic glucocorticoid, dexamethasone, has a "permissive" effect on the glucagon induction of amino acid transport in hepatocytes.

Parenchymal cells from adult rat liver were isolated by a

collagenase perfusion technique and were maintained in monolayer culture in serum-free medium on collagen coated plates as described previously[7-9]. These cells maintain many of the biochemical characteristics of adult rat liver cells for about five days in culture[7-9]. Amino acid transport was measured by studying the uptake of the nonmetabolisable amino acid α-aminoisobutyric acid (AIB).

The data presented in Table 1 established that dexamethasone, when added alone to cultures of adult rat liver parenchymal cells, has no effect on AIB transport. Experiments conducted with higher or lower concentrations of hormone and with longer or shorter times of treatment failed to demonstrate any effect of dexamethasone on amino acid transport. The addition of glucagon to the cultures resulted in a twofold increase in AIB transport. The simultaneous addition of dexamethasone and glucagon, however, produced a fourfold increase in transport. The addition of dibutyryl cyclic AMP to the cells mimicked the effect of glucagon with respect to AIB transport. The simultaneous addition of dexamethasone and dibutyryl cyclic AMP resulted in a further increase in AIB transport relative to those cells treated with dibutyryl cyclic AMP alone.

Table 1 The effect of glucagon, dibutyryl cyclic AMP, and dexamethasone alone and in combination on α-aminoisobutyrate transport

Hormone	AIB transport*
None	0.71 ± 0.02
Dexamethasone	0.70 ± 0.03
Glucagon	1.33 ± 0.06
Dexamethasone and glucagon	2.77 ± 0.11
Dibutyryl cyclic AMP	1.65 ± 0.06
Dexamethasone and dibutyryl cyclic AMP	3.43 ± 0.10

* nmol per mg cell protein per 4 min.
Adult rat liver parenchymal cells, which had been maintained in culture for 8 h, were treated with the indicated hormones for 12 h and then the uptake of 1 mM 2-^{14}C-AIB in 4 min was determined. Uptake of AIB was linear for at least 8 min. The measurement of AIB uptake was conducted in a manner similar to that for the measurement of sugar uptake in cultured cells[10]. Briefly, the procedure consisted of rinsing the cells, which were attached to 60-mm diameter dishes, with 10–15 ml of Hanks'–Hepes medium (at 37 °C and at 8 mM with respect to glucose) and 1.5 ml of this medium containing 1mM 2-^{14}C-AIB was added for 4 min at 37 °C. Incubation was terminated by aspiration of the medium and the cells were rinsed with 15–20 ml of cold phosphate buffered saline. The cells were digested in 0.2 N NaOH and samples were taken for scintillation counting and protein assay[11]. The concentrations of the hormones were as follows; dexamethasone, 10^{-6} M; glucagon, 10^{-7} M; dibutyryl cyclic AMP, 10^{-4} M. This concentration of glucagon gave the maximum induction of transport. Each value represents the mean ± s.e. of 6 plates from 3 separate experiments.

Amino acids are major substrates for gluconeogenesis in mammalian liver[5]. The alanine cycle functions to provide carbon substrate to the liver for gluconeogenesis[12,13]. The glucocorticoids have already been implicated in maintaining the supply of amino acids to the liver since these hormones tend to increase the release of amino acids from extrahepatic tissues[14-16]. The data presented in this report now establish that the glucocorticoids also play an important role in the uptake of amino acids by liver since they are required for the full induction of amino acid transport by glucagon. We believe that this is the first observation of a "permissive" effect of the glucocorticoids on the hormonal induction of a transport system.

The site of action of the "permissive" effect of the glucocorticoids is not known although RNA and protein synthesis may be required[17]. Exton *et al.*[3], have established that cyclic AMP accumulation in perfused rat liver from adrenalectomised animals after glucagon addition was normal but either hydrocortisone or dexamethasone has to be administered to observe the normal glucagon activation of gluconeogenesis. Other work[2] has also suggested that the site of the "permissive" effect is at a point beyond

cyclic AMP formation. Our data are in accord with these results since dexamethasone also had a permissive effect on the dibutyryl cyclic AMP induction of amino acid transport.

Our observation of the lack of any effect of dexamethasone alone on amino acid transport in hepatocytes in culture was rather unexpected since previous reports based on the administration of the glucocorticoids *in vivo* had suggested that these hormones alone can stimulate amino acid transport in hepatocytes[18,19]. The study of hormonal regulation of metabolic processes *in vivo* is, however, always complicated by the effects of the many endogenous hormones. Thus, the use of rat liver parenchymal cells in culture for the study of hormonal regulation of metabolic processes circumvents this problem since these cells can be maintained in a completely defined serum-free medium and still maintain many of the biochemical characteristics of adult rat liver.

ROLF F. KLETZIEN
MICHAEL W. PARIZA
JOYCE E. BECKER
VAN R. POTTER

McArdle Laboratory for Cancer Research,
University of Wisconsin,
Madison, Wisconsin 53706

1 Fain, J. N., Kovacev, V. P., and Scow, R. O., *J. biol. Chem.*, **240**, 3522–3529 (1965).
2 Schaeffer, L. D., Chenoweth, M., and Dunn, A., *Biochim. Biophys. Acta*, **192**, 304–309 (1969).
3 Exton, J. H., *et al.*, *J. biol. Chem.*, **247**, 3579–3588 (1972).
4 Ingle, D. J., *J. Endocr.*, **8**, xxiii (1952).
5 Exton, J. H., *Metabolism*, **21**, 945–990 (1972).
6 Mallette, L. E., Exton, J. H., and Park, C. R., *J. biol. Chem.*, **244**, 5724–5728 (1969).
7 Bonney, R. J., Becker, J. E., Walker, P. R., and Potter, V. R., *In Vitro*, **9**, 399–413 (1974).
8 Pariza, M. W., Becker, J. E., Yager, J. D. Jr, Bonney, R. J., and Potter, V. R., in *Differentiation and Control of Malignancy of Tumor Cells* (edit. by Nakahara, W., Ono, T., Sugimura, T., and Sugano, H.), 257–284 (University Park Press, Baltimore, 1975).
9 Pariza, M. W., *et al.*, in *Expression and Carcinogenesis in Cultured Liver* (edit. by Gerschenson, L. E., and Brad Thompson, E. B.) (Academic, New York, in the press).
10 Kletzien, R. F., and Perdue, J. F., *J. biol. Chem.*, **249**, 3366–3374 (1974).
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. biol. Chem.*, **193**, 265–275 (1951).
12 Mallette, L. E., Exton, J. H., and Park, C. R., *J. biol. Chem.*, **244**, 5713–5723 (1969).
13 Felig, P., Pozefsky, T., Marliss, E., and Cahill, G. F. Jr, *Science*, **167**, 1003–1004 (1970).
14 Bondy, P. K., Ingle, D. J., and Meeks, R. C., *Endocrinology*, **55**, 354–360 (1954).
15 Smith, O. K., and Long, C. N. H., *Endocrinology*, **80**, 561–566 (1967).
16 Exton, J. H., *et al.*, *Rec. Prog. Hormone Res.*, **26**, 411–443 (1970).
17 Braun, T., and Hechter, O., *Proc. natn. Acad. Sci. U.S.A.*, **66**, 995–1001 (1970).
18 Noall, M. W., Riggs, T. R., Walker, L. M., and Christensen, H. N., *Science*, **126**, 1002–1005 (1957).
19 Chambers, J. W., Georg, R. H., and Bass, A. D., *Molec. Pharmacol.*, **1**, 66–76 (1965).

Interference with normal phagosome–lysosome fusion in macrophages, using ingested yeast cells and suramin

FOREIGN bodies, including microorganisms, ingested by cultured mouse peritoneal macrophages become enclosed within phagosomes. Usually lysosome granules promptly assemble close to the phagosomes and fuse with their membranes, exposing the ingested objects to digestive enzymes[1–3]. Much of the kinetics of phagosome–lysosome fusion and degranulation in leukocytes was described by Robineaux, Hirsch, Cohn and their co-workers[4–7], but the mechanisms and factors controlling them are only now beginning to emerge[8,9].

Some natural exceptions to the usual fusion pattern in macrophages have been observed, notably the non-fusion response to virulent *Myobacterium tuberculosis*[10] and living *Toxoplasma gondii*[11]. These non-fusion patterns can be reversed by attaching antibody to the organisms before their ingestion[12–14]; extensive phagolysosome formation then occurs. Such deviations and manipulations of phagosome–

lysosome fusion behaviour may help to elucidate its importance in the response of macrophages to intracellular microorganisms. The examples cited have been concerned with the parasite within its phagosome, but we now report successful inhibition of the normal fusion mechanism by a "lysosomotropic" drug (suramin), using a simple rapid method for observing fusion patterns in living cultured macrophages with the light microscope. Lysosomes were prelabelled with acridine orange[15,16] and the phagocytes permitted to ingest live baker's yeast cells; intracellular changes were monitored in the living monolayers by dark-field fluorescence microscopy.
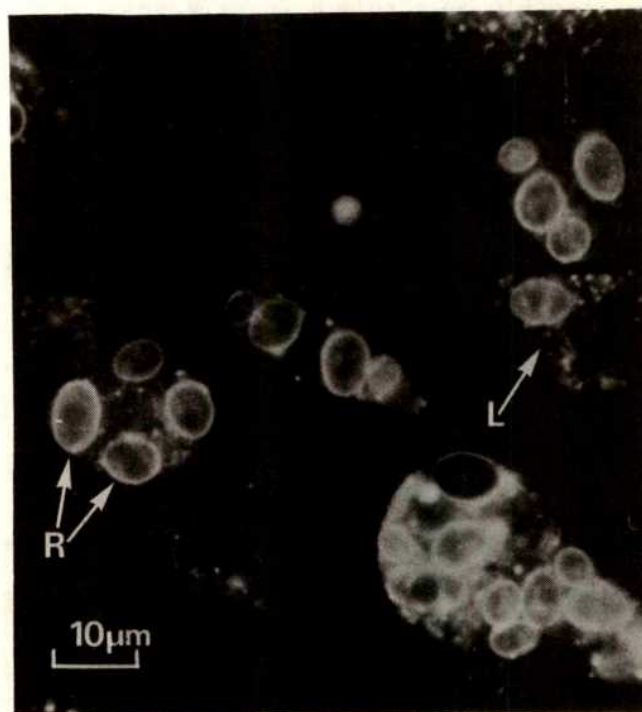
Fig. 1 Living macrophages with acridine-stained lysosomes (L) after incubation with live yeasts for 45 min. Surrounding many yeast cells are brightly fluorescent intraphagosomal "rims" (R), indicative of phagosome–lysosome fusion. Normal pattern of macrophage response.

Established cover slip monolayers of unelicited mouse peritoneal macrophages in Chang medium in Leighton tubes[10,14,17] were given a change of medium, and 2–6 d later were washed with balanced salt solution (BSS) and exposed at 37 °C in BSS to purified acridine orange, 5 μg ml^{-1}, for 10 min. The cells were washed and infected in BSS plus 2.5% human cord serum with a suspension of fresh commercial compressed baker's yeast (*Saccharomyces cerevisiae*) (about 5×10^6 yeast cells per ml; ratio to macrophages about 20 : 1). After 15 min to 6 h at 37 °C (the exposure to the yeast cells usually maintained throughout), cover slips were removed and mounted in BSS, and were examined with blue–violet light[15,16].

Lysosomes fused with yeast-containing phagosomes in characteristic stages during 1–2 h after the start of ingestion. The orange-fluorescing granules assembled around the unstained yeasts. Next, these periphagosomal "satellite" lysosomes disappeared and vivid orange confluent "rims" of fluorescence appeared around the individual yeasts, indicating a discharge of lysosomal contents into the phagosomes (see Fig. 1). Gradually the intraphagosomal dye permeated into the yeast cells, colouring the whole organisms green and then red; some hours later dark holes were sometimes seen, suggesting dissolution of yeast cells by digestive enzymes. These stages overlapped. Both satellite lysosomes and rims were plentiful at 15 min, but rims predominated at 45–50 min; coloured yeast cells increased and became

frequent after about 2 h, with gradual disappearance of rims. Heat-killed yeast cells were unsuitable for observing the fusion process as they took up the dye too rapidly for rims and satellites to be detected.

The monolayers were exposed to various substances that may inhibit the fusion process, before (or after) labelling of the lysosomes; yeast cells were then introduced. We examined some reputed stabilisers of lysosomes (dexamethasone sodium phosphate; chloroquine diphosphate; chlorpromazine hydrochloride); another anti-inflammatory agent (aspirin); an inhibitor of degranulation (colchicine) and also morphine hydrochloride, cyclophosphamide and cycloheximide. None inhibited noticeably the phagosome–lysosome fusion process in our test system.

Suramin (which enhances tuberculous infection in mouse macrophages)[17,18] was then tested. It was incorporated in fresh culture medium for 6 d; the cell layers were washed, stained with acridine orange, and infected with yeast cells as before. Uptake was normal, but 45–50 min after the start of ingestion lysosome satellites were predominant, rims and
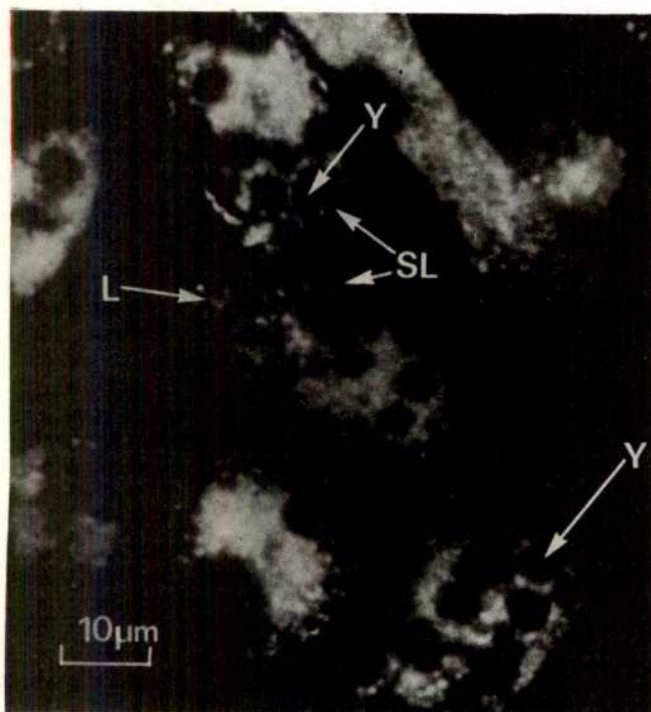
Fig. 2 Conditions as Fig. 1 but macrophages pretreated with suramin (200 μg ml^{-1} for 6 d). Lysosomes (L) have assembled as 'satellites' (SL) around many of the ingested yeast cells (Y); fluorescent rims are absent. Non-fusion pattern of response.

coloration of the yeasts being inconspicuous (Fig. 2); concurrent controls at the same time showed mostly rims and some green or red yeast cells (Fig. 1). These appearances were interpreted as almost complete short term suppression of the normal phagosome–lysosome fusion response. They were evident after pretreatment at 200 μg ml^{-1} (maximum tolerated) to 50 μg ml^{-1}, with some inhibition down to 5 μg ml^{-1}. The effect was maintained for at least 5 h after 200 and 100 μg ml^{-1}; however, after 50 μg ml^{-1} a few rims and coloured yeast cells appeared towards the end of this period. The drug at 200 μg ml^{-1} only partly suppressed the fusion response to heat-killed yeast cells. The suppression of fusion by suramin was confirmed by electron microscopy, prelabelling secondary lysosomes with ferritin.

The macrophage monolayers were pulsed with ferritin (10 mg ml^{-1}) for 3 h in Leighton tubes[14]. Next day, suramin (200 μg ml^{-1}) was added to fresh medium in half the tubes and all were incubated for 6 d at 37 °C. After washing, live yeasts were added and the tubes reincubated, and the macrophages were fixed 40–50 min later[10,19].
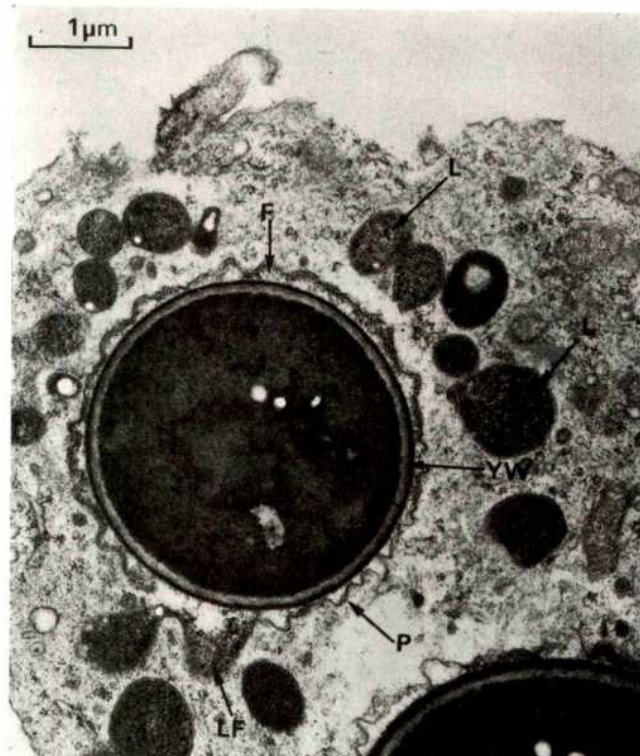
Fig. 3 Ultrastructure of a macrophage with ferritin-labelled secondary lysosomes (L) after incubation with live yeast cells for 45 min. Ferritin label (F) surrounds a yeast cell wall (YW) inside the 'tight' phagosome membrane (P), indicating phagosome–lysosome fusion. A lysosome is seen fusing (LF).

In thin sections of controls fixed 45 min after the start of ingestion (Fig. 3), ferritin label was seen within about 80% of yeast-containing phagosomes. The phagolysosomal membrane was invariably applied closely to the surface of the enclosed yeast cell, that is, of a "tight" variety[20,21]. About
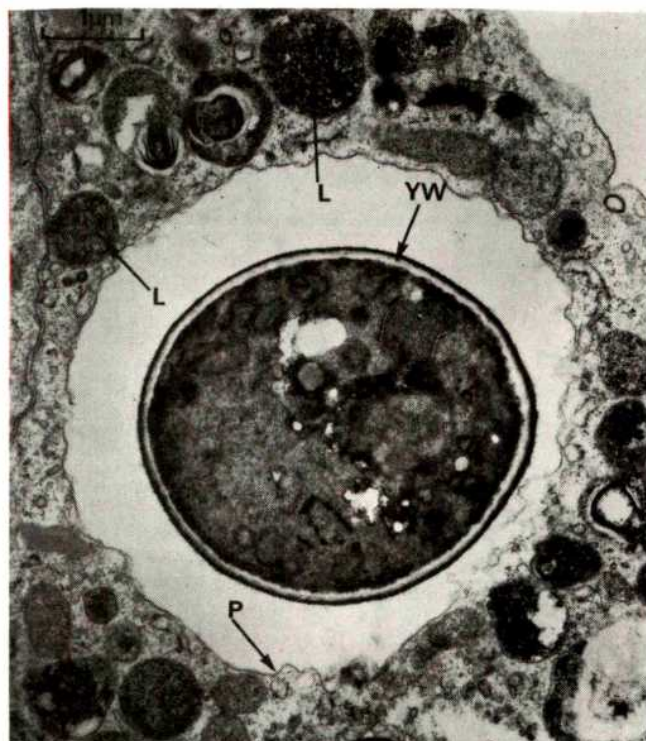
Fig. 4 Conditions as Fig. 3 but macrophages pretreated with suramin (200 μg ml^{-1}). Lysosomes (L) are labelled but ferritin is absent from zone between yeast wall (YW) and 'loose' phagosome membrane (P). Non-fusion.

half the yeasts were "granular" in appearance, suggesting incipient degradation. In suramin-pretreated macrophages (Fig. 4) the ferritin label had entered only about 10% of phagosomes; where fusion had not occurred, the phagosome membranes were almost all of a "loose" variety[20,21]. The yeast cells mostly appeared structurally intact. Autophagic vacuoles were present in a proportion of the macrophages pretreated with suramin at 200 μg ml^{-1}.

We infer that suramin does not interfere with the peri-phagosomal assembly of lysosomes, but affects the membrane fusion. Suppression of this process in leukocytes by this trypanocide has not previously been reported, and it is not clear whether any of its known properties (see refs 22–25) are relevant. Suramin is a polybasic anion and binds strongly to plasma proteins. It enters cells by endocytosis and becomes concentrated in their lysosomes. It inhibits many enzymes, including some of the lysosomal proteases, and it also inhibits (Na$^+$−K$^+$)-activated ATPase. It is anti-complementary.

The live-yeast/acridine orange system may prove useful for studying the mechanisms of phagosome–lysosome fusion in cultured macrophages, and throw light on how non-pathogenic microorganisms are inactivated and disposed of —especially whether the lysosomal enzymes actually kill, or merely digest organisms inactivated by other processes. Manipulations by means of a fusion inhibitor such as suramin should add to the usefulness of such a system.

We thank Dr J. A. Armstrong for valuable advice and criticism; and Drs D. H. Williamson and J. Williamson for technical suggestions relating, respectively, to yeast cells and suramin.

P. D'Arcy Hart
M. R. Young

*National Institute for Medical Research,
Mill Hill, London NW7 1AA, UK*

1 Trouet, A., *Bruxelles-Médical, Rev. Belge Sci. Méd. Chir.*, **46**, 1139–1144 (1966).
2 Axline, S. G., and Cohn, Z. A., *J. exp. Med.*, **131**, 1239–1260 (1970).
3 Jensen, M. S., and Bainton, D. F., *J. Cell Biol.*, **56**, 379–388 (1973).
4 Robineaux, J., and Frederic, J., *C. r. Soc. Biologie*, **149**, 486–489 (1955).
5 Hirsch, J. G., and Cohn, Z. A., *J. exp. Med.*, **112**, 1005–1014 (1960).
6 Hirsch, J. G., *J. exp. Med.*, **116**, 827–834 (1962).
7 Cohn, Z. A., and Wiener, E., *J. exp. Med.*, **118**, 1009–1020 (1963).
8 Poste, G., and Allison, A. C., *Biochim. biophys. Acta*, **300**, 421–465 (1973).
9 Ahkong, Q. F., Fisher, D., Tampion, W., and Lucy, J. A., *Nature*, **253**, 194–195 (1975).
10 Armstrong, J. A., and Hart, P. D'Arcy, *J. exp. Med.*, **134**, 713–740 (1971).
11 Jones, T. C., and Hirsch, J. G., *J. exp. Med.*, **136**, 1173–1194 (1972).
12 Jones, T. C., *J. reticuloendothel. Soc.* **15**, 439–450 (1974).
13 Jones, T. C., Len, L., and Hirsch, J. G., *J. exp. Med.*, **141**, 466–482 (1975).
14 Armstrong, J. A., and Hart, P. D'Arcy, *J. exp. Med.* (in the press).
15 Allison, A. C., and Young, M. R., *Life Sci.*, **3**, 1407–1414 (1964).
16 Robbins, E., Marcus, P. I., and Gonatas, N. K., *J. Cell Biol.*, **21**, 49–62 (1964).
17 Hart, P. D'Arcy, *Science*, **162**, 686–689 (1968).
18 Rees, R. J. W., and Hart, P. D'Arcy, *Tubercle*, **37**, 327–332 (1956).
19 Hirsch, J. G., and Fedorko, M. E., *J. Cell. Biol.*, **38**, 615–627 (1968).
20 Dumont, A., and Robert, A., *Lab. Invest.*, **23**, 278–286 (1970).
21 Belcher, R. W., Carney, J. F., and Monahan, F. G., *Lab. Invest.*, **29**, 620–627 (1973).
22 Williamson, J., in *The African Trypanosomiases*, 172–174 (Allen and Unwin, London, 1970).
23 Eisen, V., and Loveday, C., *Br. J. Pharmac.*, **49**, 678–687 (1973).
24 Fortes, P. A. G., Ellory, J. C., and Lew, V. L., *Biochim. biophys. Acta*, **318**, 262–272 (1973).
25 de Duve, C., *et al.*, *Biochem. Pharmac.*, **23**, 2495–2531 (1974).

Protective tumour cell ghosts with intact membrane markers

TRANSPLANTATION antigen-like tumour-associated surface membrane antigens (TATA) have now been prepared in solubilised or semi-solubilised form[1-3]. The methods of preparation are, however, complicated and yields are usually poor. Moreover, protection experiments with solubilised tumour antigens have rarely been successful and have given very limited protection[1,2,4,5]. On the other hand, all manner of devitalised cells protect in immunisation experiments against living tumour cells[6]; and it is therefore possible that interference with cell membrane integrity during solubilisation destroys tertiary and quarternary structures important for the immunogenicity of membrane antigens[6,7]. We describe here a simple and gentle procedure for preparing in near-quantitative yield (nucleated) tumour cell 'ghosts' with retention of protective immunogenicity. Freeze drying of 'ghost' suspensions provided a stable material which retained its protective activity for 2.5 yr when stored dry at 4 °C.

Ehrlich ascites tumour cells (EATC) were obtained from BALB/c mice inoculated intraperitoneally 8–9 d previously with 10^6 viable (dye exclusion) EATC and were immediately mixed with an equal volume of Hanks' balanced salt solution (HBSS) containing 10^{-3} M EDTA, thus giving an homogeneous suspension essentially free of erythrocytes (RBC). After two washes in HBSS or Tyrode (5 min centrifugation at 1,000g followed by resuspension to the original volume of fresh solution), the cells were subjected to hypotonic shock, essentially as performed by Ponder in the preparation of RBC ghosts[8]; the EATC were suspended at a concentration of 10^8 ml^{-1} in CO$_2$ saturated water at 4 °C, held at this temperature for 10 min, centrifuged and resuspended in fresh hypotonic solution for another 10 min. After repeating this sequence, the suspension was washed four to five times with buffer (0.14 M NaCl in 0.01 M Tris, pH 7.2). Table 1 shows that about 50% of the cell protein is extracted together with all the EATC glucose-6-phosphate dehydrogenase, an enzyme known to be associated

Table 1 Recoveries of ghosts from EATC in terms of cell counts and comparisons of proteins and enzyme contents of ghosts and intact cells

	Intact EATC	Ghosts	% Recovery
Total cell counts ($\times 10^{-9}$)	1.44±0.08	1.34±0.09	93
Protein (mg per 10^9 cells)	211±21	95±30	45
Total ATPase*	191±41	194±43	100
5'-nucleotidase*	27±4	24±5	100
Glucose-6-phosphate dehydrogenase†	1.82±0.41	Nil	Nil

Ghosts can be counted as readily as intact cells and would be regarded as viable by dye exclusion although they do not form tumours when injected into mice.

*μmol phosphate liberated per h per 10^9 cells.
†μmol NADPH produced per min per 10^9 cells.

with cell cytoplasm[9]. On the other hand, ATPase$^+$ and Na$^+$ K$^+$ ATPase, enzymes occurring in association with membranes[10-12], were retained quantitatively in the ghosts. Although 5'-nucleotidase has been shown to be membrane-bound in rat liver[11,12], in EATC its activity proved to be too low to be of value as a membrane marker.

Phase contrast microscopy of the EATC 'ghosts' showed a slightly enlarged and still nucleated cell with an otherwise 'empty bag' appearance. Acridine orange (AO) staining[13,14] (using two drops of cell suspension with two drops of a 0.02%

Table 2 Quantification of membrane immunofluorescence produced by reacting fluorescein-labelled goat anti-mouse Ig with known numbers of EATC (or ghosts) incubated previously with rabbit anti-EATC serum

Rabbit anti-EATC (dilution)	Fluorimetric readings		
	Original EATC	Ghosts	Control
1:5	0.95±0.05	0.96±0.05	0.03
1:10	0.82±0.04	0.80±0.05	0.03

5 × 10^6 cells were used per test and the carefully washed fluorescent cells were finally suspended in 2 ml Tris–Triton X-100. Control cells were treated with equivalent dilutions of normal rabbit serum in place of the rabbit anti-EATC serum. Fluorimetric readings were taken within 1 h of completing the tests.

aqueous solution of AO) showed under ultraviolet light that the ghosts had retained the green-fluorescing nucleus of normal EATC. The plasma membrane and a fibre-like fine network in the cytoplasm could be seen in dull red-brown outline. The usually bright orange-fluorescing cytoplasmic granules of intact EATC, however—most likely lysosomes—had disappeared, confirming the leakage of cytoplasmic contents already indicated by the chemical and enzymic tests. Yet the original cell shape was well maintained in ghosts stored in the isotonic Tris-NaCl buffer. This, in conjunction with retention of membrane enzymes, suggests remarkable retention of structural integrity and retention of an at least partially intact membrane system. Freeze-dried ghosts had a somewhat crumpled appearance, but still showed the essential structural features.

The epitopic antigen densities of the ghosts were compared with those of the intact cells by a quantitative membrane immunofluorescent method (R.A. and L. Waft, unpublished). Briefly, aliquots of 5×10^6 washed EATC (or ghost equivalents) were first reacted with rabbit anti-EATC serum and, after three washes in Hanks' balanced salt solution (centrifugation at $1{,}000g$ for 5 min), with fluorescein-conjugated anti-rabbit IgG. After further washings the cells were finally suspended in Tris–Triton X buffer (a mixture of equal volumes of 1% Triton X-100 in water and 0.04 M Tris-HCl, pH 8.0). This yields a stable fluorescent cell suspension which can be read in a conventional fluorimeter (Aminco–Bowman). Table 2 shows that equivalent numbers of intact EATC and EATC 'ghosts' gave identical readings, whereas controls (immune serum replaced by an equivalent dilution of normal rabbit serum) gave readings of about 3% of the test readings. These tests therefore indicate retention of the full surface complement of antigens demonstrable by the rabbit anti-EATC serum used.

Retention of protective anti-EATC surface immunogenicity was further established by immunising three groups of mice respectively with X-irradiated but otherwise untreated EATC (E*)[6,15], with ghosts and with the extract concentrates from preparing the ghosts (Ex). Each mouse received two spaced (2 weeks) injections of 10^8 E* or ghosts, and the third group the extracts of twice that number of EATC. One week after the second injection all the mice were challenged with freshly collected tumour cells (viability >95% by dye exclusion), either EATC or cells from an unrelated tumour (methylcholanthrene-induced, MC). Table 3 shows that immunisation with EATC ghosts protected as well as did the E*, whereas the extracts of even twice the number of EATC provided no protection whatever. Table 3 shows that the unrelated tumour cells grew as well in the EATC-injected as in the uninjected control mice. The specificity of the reaction was confirmed by lymphoid cell transfer experiments[16]. Draining node cells from mice injected with E*, ghosts or Ex were collected 5 d after the last subcutaneous injection, mixed with 5×10^5 EATC or 10^5 MC tumour cells (lymphoid cell–tumour cell ratio 100:1) and injected subcutaneously into groups of normal mice. The control groups received the two types of tumour cells

Table 3 Protective immunisation in BALB/c mice

Immunising dose (Cell no. per subcutaneous injection)	Challenge (No. of viable tumour cells interperitoneally)	Tumour takes in:	
		Test	Control
10^8 E*	10^6 EATC	0/12	6/6
10^8 E*	10^5 MC	6/6	6/6
10^8 ghosts	10^6 EATC	1/18	6/6
10^8 ghosts	10^5 MC	6/6	6/6
Ex from 2×10^8 EATC	10^6 EATC	11/12	6/6
Ex from 2×10^8 EATC	10^5 MC	5/6	5/6

E*, X-irradiated EATC (10,000 rad).
Ex, combined concentrated EATC extracts obtained during the preparation of EATC ghosts.
MC, methylcholanthrene-induced tumour cells.

Table 4 Lymphoid cell transfer tests

Lymphoid cells from mice immunised with:	Admixed tumour cells	Tumour takes in:	
		Test	Control
E*	5×10^5 EATC	1/12	6/6
Ghosts	5×10^5 EATC	0/6	6/6
Ghosts	10^5 MC	6/6	6/6
Ex	5×10^5 EATC	6/6	3/3

Lymphoid to tumour cell ratio in the injected mixtures, 100:1.

alone. Table 4 shows that EATC tumour formation, but not MC tumour formation, was prevented by lymphoid cells from mice immunised with E* or EATC ghosts. As expected, lymphoid cells from mice immunised with Ex failed to prevent EATC tumour formation.

The fact that an essentially cytoplasm-free nucleated cell membrane bag has been found quantitatively to retain surface antigens of the intact cell and has proved fully immunogenic in protection experiments, whereas the cytoplasmic cell sap has proved completely inactive, suggests that (at least in EATC) TSTA (or TATA) are closely and probably exclusively associated with the plasma (and possibly other) cell membranes, whereas the cytoplasm is free of TSTA (TATA). Ghosts of the type prepared here may prove useful not only for protective immunisation, but also as a more satisfactory starting material than intact cells in cell-antigen purification. A fully immunogenic intermediate of this type may also help clarify features involved in preserving protective tumour immunogenicity during purification.

I. ab I. Davies*
M. G. Nicklin†
R. Augustin

Sub-department of Immunology,
Liverpool University,
Crown Street, PO Box 147,
Liverpool L69 3BX, UK

1 Meltzer, M. S., Leonard, E. J., Rapp, H. J., and Borsos, T., *J. natn. Cancer Inst.*, 47, 703 (1971).
2 Baldwin, R. W., and Glaves, D., *Clin. exp. Immun.*, 11, 51 (1972).
3 Kedar, E., Aronov, A., Sulitzenau, D., *Israel J. med. Sci.*, 9, 266 (1973).
4 Law, L. W., and Appella, E., *Nature*, 243, 83 (1973).
5 Holmes, E. C., Kahan, B. D., and Morton, D. C., *Cancer*, 25, 373 (1970).
6 Augustin, R., *Nature*, 224, 295 (1969).
7 Wolf, A., and Avis, P. J. G., *Transplantation*, 9, 18 (1970).
8 Ponder, E., *J. exp. Biol.*, 18, 257 (1942); in *The Cell*, 2 (edit. by Brachet, J., and Mirsky, A. E.), (Academic, New York, 1961).
9 Dodge, J. T., Mitchell, C., and Hanahan, D. J., *Archs Biochem. Biophys.*, 100, 119 (1962).
10 Wallach, D. F. H., and Ullrey, D., *Biochim. biophys. Acta*, 64, 526 (1964).
11 Emmelot, P., and Bos, C. J., *Biochim. biophys. Acta*, 59, 375 (1966).
12 Bos, C. J., and Emmelot, P., *Int. J. Cancer*, 4, 705 (1969).
13 Fitzpatrick, M. E., et al., *Immunology*, 12, 1 (1967).
14 West, C. R., and Augustin, R., in *Cytology Automation* (edit. by Evans, D. M. D.), 121 (Proc. second Tenovus Symp., Livingstone, 1970).
15 Augustin, R., West, C. R., Sparshott, S. M., and Chandradasa, K. D., in *Immunity and Tolerance in Oncogenesis*, 2 (edit. by Severi, L.), 931 (Perrugia University Press, Italy, 1970).
16 Winn, H. J., *J. Immun.*, 86, 228 (1961).

Enhanced immunological surveillance in mice heterozygous at the H-2 gene complex

THE major histocompatibility (H) antigens of higher animals show extreme genetic polymorphism equalled, in higher vertebrates, only by that associated with the immunoglobulins[1]. Maintenance of such a high rate of variability implies

evolutionary advantage for heterozygotes in the HL-A system for man, or at the H-2 gene complex in mice[2,3]. We propose a possible selective mechanism, based on the realisation that immunological surveillance function (defined here as recognition and elimination of modified host cells by sensitised thymus-derived lymphocytes (T-cells) may be considerably enhanced in mice heterozygous at the H-2 gene complex.

This idea arose from experiments with two of the most prevalent naturally occurring virus infections of mice, lymphocytic choriomeningitis (LCM) and ectromelia (mouse pox). In both diseases immune T cells seem to be sensitised to altered self antigens[4-7], the self components involved being specified at, or near to, either of the two loci (H-2K or H-2D) coding for the major transplantation antigens. Whether viral and H-2 components are in some way complexed, or more long range modification of H-2 antigens is induced by the infectious process, is neither known nor central to the present argument.

Table 1 Cytolytic activity of LCM-immune spleen cells for H-2[k] virus-infected L cells, or L-929 fibroblasts

Mouse strain	H-2K	H-2D	Lytic units*	% Activity
CBA/H	kk	kk	6.5×10^4	100
CBA/H × C57BL F$_1$	kb	kb	9.0×10^4	72
B10.A	kk	dd	16.0×10^4	40
C57BL	bb	bb	$> 1,000 \times 10^4$	< 1

* Number of LCM-immune spleen cells necessary to cause 33% specific ^{51}Cr release from 5×10^4 (cells per assay well) LCM virus-infected targets[4,8]. Mice were injected intravenously with 2,000 LD$_{50}$ WE3 LCM virus 8 d before.

The evidence for this 'altered self' hypothesis is as follows[4-7]: First, lysis of LCM virus-infected targets by sensitised T cells occurs only if the two cell types are compatible for either one parental H-2 haplotype, or at H-2K or H-2D. Identity of immune response genes (I region) is neither sufficient nor necessary. Second, results of selective proliferation studies *in vivo* and 'cold-target' competitive inhibition experiments *in vitro* indicate that F$_1$ mice generate LCM-immune T cells of at least two specificities, each directed against altered self characteristics of one parental H-2 haplotype. Third, similar experiments using H-2 recombinant mice also show that homozygotes possess a minimum of two T cell specificities, associated with H-2K or H-2D. Heterozygotes would thus generate T cells of at least four specificities, sensitised to altered self antigens coded for at the H-2K and H-2D loci of each parental haplotype. The total range of the T cell response in the F$_1$ may thus be

Table 3 Susceptibility of H-2 different mice sharing the C57BL genetic background

	log$_{10}$ intracerebral virus dose	B10.A (H-2[a])	B10.A × C57BL F$_1$	C57BL (H-2[b])
% Mortality*	3	10	40	10
	2	10	60	30
	1	10	90	60
Days to death	3	9.5	8.5 ± 0.6	11.5
	2	10.0	7.5 ± 0.3†	8.5 ± 0.6
	1	9.0	7.9 ± 0.3‡	9.3 ± 0.1

* Groups of 10 adult ♂ mice were used.
†, $P > 0.05$.
‡, $P < 0.001$.

as much as twice that possible in either parental homozygote.

Comparison of cytotoxic T cell activities *in vitro* provides support for this concept. Repeated experiments have shown that compatibility (between T cell and target cell) at one H-2 haplotype confers at least 70% of the cytolytic capacity found in the homozygous situation[4] (that is, full compatability between homozygous killer T cell and target cell). Results for some of the mouse strains used in the *in vivo* experiments described below are shown in Table 1. The possible response in the F$_1$ associated with both haplotypes could thus be in excess of 140% of that occurring in either parent.

Enhanced T cell responsiveness in F$_1$ mice may also be shown *in vivo*. The LCM model used here is paradoxical in so far as our general argument is concerned, as it reflects a fatal immunopathological process directed against virus-infected cells in the central nervous system. The same response to virus growing in lung and liver results in immune elimination and recovery[9], the normal consequence of cell-mediated immunity[10]. Mice injected intracerebrally with a low dose of viscerotropic (WE3) LCM virus die with severe neurological symptoms in 7–9 d. Death seems to reflect damage to virus-infected meningeal and choroid plexus cells caused by H-2 compatible immune T cells[5,11], LCM virus itself being of minimal pathogenicity. Inoculation of a large dose of viscerotropic virus intracerebrally, however, often results in survival. An explanation for this seemingly high dose paralysis[12,13] is that administration of large amounts of virus leads to more extensive growth in viscera, with resultant recruitment of sensitised T cells to peripheral sites and reduced neuropathology mediated by T cells.

Heterozygotes were consistently more susceptible than were parental homozygotes to intracerebral inoculation with WE3 LCM virus, the effect being apparent at all dose

Table 2 Susceptibility of parental and F$_1$ mice to intracerebral WE3 LCM virus

	log$_{10}$ intracerebral virus dose	BALB/c (H-2[d])	BALB/c × C57BL F$_1$	C57BL (H-2[b])	C57BL × CBA/H F$_1$	CBA/H (H-2[k])
% Mortality	3*	25	100	40	100	40
	4	13	100	40	100	63
	3	88	100	13	100	88
	2	100	100	88	100	100
	1	63	100	100	100	100
Days to death	3*	7.3 ± 0.3†	8.0 ± 0.3§	10.3 ± 0.6¶	7.1 ± 0.1§	7.6 ± 0.1
	4	8.5	7.6 ± 0.4§	11.0 ± 0.9¶	7.2 ± 0.2¶	10.7 ± 0.9
	3	8.7 ± 0.4§	7.5 ± 0.2	13.0	7.1 ± 0.2†	9.6 ± 1.0
	2	9.2 ± 0.4§	7.8 ± 0.1¶	11.0 ± 0.5¶	7.3 ± 0.2†	7.8 ± 0.1
	1	8.8 ± 0.3‡	8.1 ± 0.1¶	10.7 ± 0.6¶	7.9 ± 0.2‡	8.4 ± 0.1

* Preliminary experiment, in which 90% of CBA/H × BALB/c F$_1$ mice died in 8.1 ± 0.2 d. The other assays were carried out concurrently. Groups of 8–10 adult (7–9 weeks) female mice were used throughout, and mice were examined twice daily for 14 d. Virus titre was determined in outbred WEHI mice, which are susceptible to intracerebral WE3 LCM virus at all dose levels.
Differences between parent strain and F$_1$ mice: † $P > 0.05$; ‡ $P < 0.05$; § $P < 0.01$; ¶ $P < 0.001$.

Table 4 Severity of meningitis following intracerebral LCM virus

	$\log_{10}$ cells per μl CSF on day:		
	6	7	8
BALB/C	3.6 ± 0.2	4.2 ± 0.1	4.2 ± 0.1
BALB/C × C57BL F_1	$4.2 \pm 0.1\ddagger$	$5.0 \pm 0.1\S$	NS
C57BL	$3.6 \pm 0.2\ddagger$	$4.1 \pm 0.1\S$	4.1 ± 0.1
C57BL × CBA/H F_1	$4.2 \pm 0.1\ddagger$	*$4.6 \pm 0.1\S$	NS
CBA/H	$3.4 \pm 0.2\ddagger$	$4.3 \pm 0.1\dagger$	4.1 ± 0.1

Mice were injected intracerebrally with 10^3 LD_{50} of WE3 LCM virus. Samples of cerebrospinal fluid (CSF) were obtained from the cisterna magna, stained with WBC diluting fluid and counted. CSF of CBA/H mice injected intracerebrally with diluent 6 d previously contains < 100 cells μl^{-1} (ref. 5).
NS, Not sampled, as no mice surviving.
* In 8 of 12 mice sampled brain swelling, the severity of which is directly related to progression of both symptoms and the inflammatory lesion[5], was so advanced that CSF could not be obtained.
Differences between parental and F_1 mice: $\dagger$, $P < 0.02$; $\ddagger$, $P < 0.01$; $\S$, $P < 0.001$.

levels tested (Tables 2 and 3). Furthermore, the inflammatory response, which is largely produced by T cells and is directly related to severity of symptoms[4,5], was both more rapid and of greater magnitude in F_1 mice (Table 4). This somewhat artefactual system serves, therefore, to illustrate that the T-cell response to virally modified cells is enhanced in heterozygotes.

Results from both *in vitro* and *in vivo* systems thus support the concept that immune responsiveness is enhanced by involvement of a greater number of T-cell specificities, directed against altered self antigens coded for at H-2K or H-2D. Such an argument provides a mechanistic basis for evolutionary advantage of both gene duplication and heterozygosity at the H-2 gene complex, in the absence of positive selection for any particular H-2 haplotype.

PETER C. DOHERTY
ROLF M. ZINKERNAGEL

Department of Microbiology,
John Curtin School of Medical Research,
Canberra, ACT, Australia

1 Amos, D. B. et al., Fedn Proc., 31, 1087–1104 (1972).
2 Bodmer, W. F., Nature, 237, 139–145 (1972).
3 Burnet, F. M., Nature, 245, 359–361 (1973).
4 Zinkernagel, R. M., and Doherty, P. C., J. exp. Med., 138, 1266–1269 (1973); Nature, 248, 701–702; 251, 547–548 (1974); J. exp. Med. (in the press).
5 Doherty, P. C., and Zinkernagel, R. M., Transplant. Rev., 19, 89–120 (1974); J. Immun., 114, 30–33 (1975); J. exp. Med., 141, 502–507 (1975).
6 Gardner, I., Bowern, N. A., and Blanden, R. V., Eur. J. Immun. (in the press).
7 Blanden, R. V. et al., Nature, 254, 269–270 (1975).
8 Cerottini, J. -C., and Brunner, K. T., Adv. Immun., 19, 67–132 (1974).
9 Mims, C. A., and Blanden, R. V., Infect. Immun., 6, 695–698 (1972).
10 Blanden, R. V., Transplant. Rev., 19, 56–88 (1974).
11 Cole, G. A., Nathanson, N., and Prendergast, R. A., Nature, 238, 335–337 (1972).
12 Hotchin, J., Mongr. Virol., 3, 1–211 (1971).
13 Doherty, P. C., Zinkernagel, R. M., and Ramshaw, I. A., J. Immun., 112, 1548–1552 (1974).

Specific basophil hypersensitivity induced by skin testing and transferred using immune serum

INTRADERMAL skin testing of appropriately immunised guinea pigs results in specific hypersensitivity reactions of the 24 h delayed type. As controls, we routinely skin test non-immune animals. We have found that skin testing itself can cause specific sensitisation for delayed time course reactions in normal guinea pigs. Skin testing non-immunised animals with the protein antigen keyhole limpet haemocyanin (KLH) leads, within several days, to striking macroscopic lesions at these primary (1°) injection sites in all animals. We have termed these responses 'flare reactions', and have found on histological examination that they contain large accumulations of basophils. Overall, these flares resemble hypersensitivity reactions with a delayed time

course termed 'cutaneous basophil hypersensitivity' (CBH)[1]. As proof that skin testing *per se* can specifically sensitise, we obtained serum from animals with these cutaneous basophil flare reactions and carried out intravenous transfers to non-immune recipients who then received a KLH skin test. Delayed 24 h CBH reactions which were specifically elicited by this native protein antigen, were uniformly transferred by immune serum.

Two separate preparations of KLH (Pacific Bio-Marine, Venice, California) were used for these studies. Hartley strain albino guinea pigs (250–300 g) were bred at the Division of Animal Care at Yale University School of Medicine. On day 0 animals were flank skin tested by intradermal injection of 200 μg KLH in phosphate buffered saline (0.1 ml) at one site. These primary injection sites were observed daily and macroscopic skin reactions were noted at 4 d in half of the animals, were present in all 40 of the animals at 5 d, and reached a peak at 6 d (Fig. 1). These reactions were erythematous, indurated, and seemed tender to the touch. Animals initially injected with 20 and 200 μg KLH at separate sites also had macroscopic reactions at the 20 μg site, which were maximal 6 d after intradermal injection (Fig. 1).
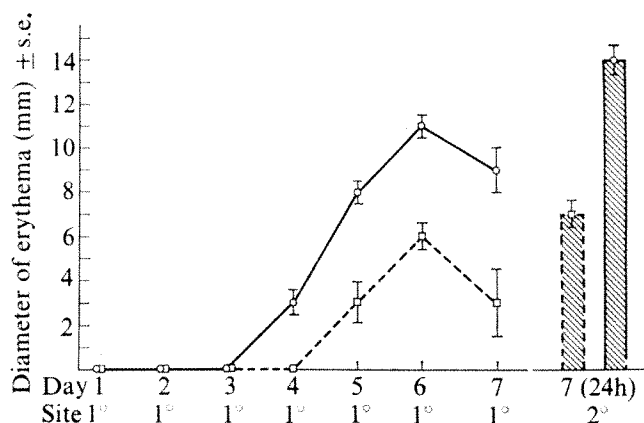
Fig. 1 Guinea pig macroscopic skin reactions at sites injected with KLH: □, 20 μg; ○, 200 μg. Primary (1°) intradermal injections were at day 0, and were read daily. Secondary (2°) injections were at day 6 and were read 24 h later.

Secondary (2°) skin tests with 20 and 200 μg KLH on the contralateral flank at day 6, resulted in large erythematous and weakly indurated reactions which began at about 4–6 h and reached a peak at 12–24 h. At this time, the 7-d-old 200 μg priming site occasionally showed superficial crusting and sloughing, and less erythema and induration than on the previous day. Histologically, this primary site (Fig. 2) and contralateral 24 h secondary skin test reactions to 200 or 20 μg KLH all had large infiltrates of basophils (Table 1) in addition to many mononuclear cells and some eosinophils. The site of skin testing performed 7 d previously with 20 μg KLH also showed CBH. It was concluded that intracutaneous injection of KLH sensitised guinea pigs for CBH, and that sufficient quantities of this antigen may have been retained at the skin test site to result in a flare of CBH at the priming site.

Table 2 shows the results of four separate experiments demonstrating serum transfer of CBH using donor animals which were immunised by intradermal injection of KLH and manifested cutaneous basophil flare reactions. Three different serum pools resulting from bleeding 7 d after priming were used for intravenous transfer of 2 ml per recipient. Two hours after transfer, recipients were skin tested with 200 μg KLH. In all four experiments, examination of these test sites showed that KLH immune sera from skin test-sensitised animals transferred macroscopic delayed (24 h) reactions containing large basophil infiltrates. Suc-

Table 1 Cutaneous basophil flares and 24 h cutaneous basophil reactions induced by intradermal injection of KLH

Time after initial injection (No. of animals)	Skin test dose	Macroscopic erythema (mm)±s.e.	Microscopic basophils* ±s.e.
Flare reactions at priming site:			
24 h (16)	200 µg	0	4±1
48 h (6)	200 µg	0	3±1
7 d (7)	200 µg	9±1	203±37
7 d (4)	20 µg	4±2	71±23
24 h CBH reactions at 6 d secondary skin test site:			
7 d (13)	200 µg	14±1	103±20
7 d (10)	20 µg	7±1	85±18

*Skin reactions were fixed in Helly's solution, embedded in paraffin and Giemsa stained for basophil identification[2]. Basophils were counted in five central and adjacent 180 µm diameter oil immersion (×1,000) fields at the uppermost dermis.

cessful CBH transfers were also accomplished with 1 week sera from KLH-primed donors which did not receive secondary skin tests. Control recipients of KLH immune sera which were intradermal skin tested with sheep red blood cells (SRBC) (5% or 20%) or irrelevant proteins (oxazalone or picryl human albumin, 200 µg) showed no macroscopic reactions and no basophil infiltrates. KLH skin tests (200 µg) in a variety of controls which received heterologous immune sera, media with 10% foetal calf serum (FCS), or nothing, also caused no macroscopic reactions and no basophil accumulations at 24 h (Table 2). It was concluded that skin tests can cause specific sensitisation for a reaction of the delayed type, that serum transfers of CBH to a native protein antigen can be achieved; and that serum factors contribute to cutaneous basophil flare reactions.

Our work has pointed out that serum factors are important in some delayed reactions containing significant basophil infiltrates. Thus, a component of some delayed basophil-containing reactions is hapten-specific[2,3], and is transferable by small quantities of immunochemically purified antibody recovered in 7S IgG$_1$ column fractions[4]. Antibody alone, however, is not the sole factor responsible for all delayed basophil infiltrates. We have shown that carrier-specific basophil accumulations are probably mediated by T cells[5]. Thus, B cell-derived antibodies and T cells probably function in the arrival of basophils in delayed cutaneous reactions.

Our previous studies of serum-transferred hapten-specific CBH necessarily involved sensitisation and testing with synthetic haptens conjugated to proteins and also used immunisation with adjuvants. In the present study, CBH sensitisation and accompanying cutaneous basophil flares were achieved by intradermal injection of a native protein without adjuvants. Serum obtained from these animals uniformly transferred specific delayed reactions, which were macroscopically evident in the flank skin of recipient guinea pigs, and showed numerous basophils on microscopic examination.

Jones and Mote originally described the ability of skin tests to sensitise humans for delayed reactions, and they noted flares at the primary site in nearly one third of individuals immunised by intradermal injections of hetero-

logous proteins[6,7]. This study with guinea pigs has shown that cutaneous flares at sites of intradermal immunisation are characterised by marked basophil accumulations; we have also noted flares of basophils containing cutaneous reactions following intradermal sensitisation of humans with KLH (P.W.A. and J. E. Atwood, unpublished). In human contact reactions, which also have basophil accumulations[1], similar flares occur at primary sensitisation sites, and flares at human skin sites primed with dinitrochlorobenzene are considered the strongest criterion of normal immune reactivity[8].
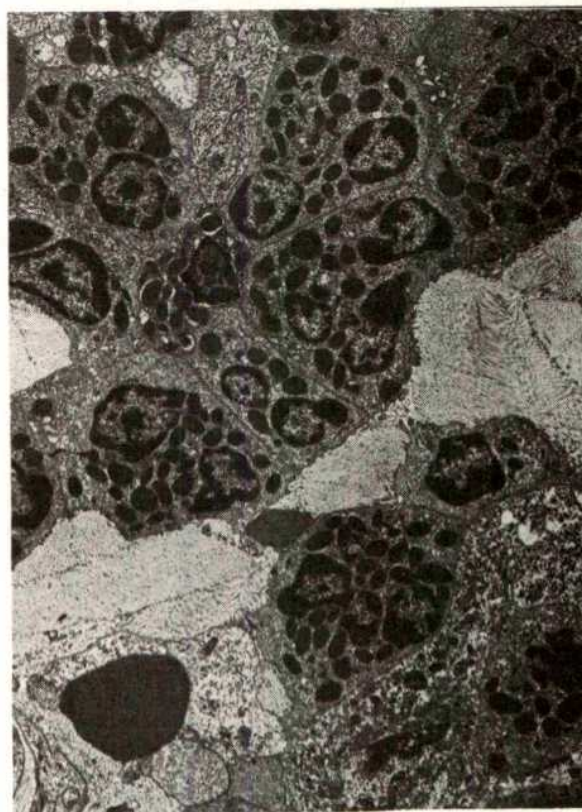
Fig. 2 Electron photomicrograph from a primary skin test site from a non-immune guinea pig injected 7 d previously with 200 µg KLH. A dense infiltrate of basophils is seen in the upper dermis. ×4,200.

A consideration of the ability of skin tests to sensitise is pertinent to the interpretation of specific immune reactivity following transfer of serum, cells or cell extracts (such as transfer factor, which may transfer specific immunity or act as an adjuvant[9]). It is possible that a successful transfer may in some instances depend on the ability of the transfer to augment and accelerate the sensitising or irritating potential[10] of the skin test used to demonstrate the transfer. In systems in which flares can develop from skin testing, interpretation of the specificity of passively acquired

Table 2 Serum transfer of CBH to KLH following intradermal (i.d.) immunisation

Serum donor immunisation	Number of: Experiments	Recipients	Material transferred	Recipient 24 h skin test reaction (200 µg KLH) Erythema (mm)	Basophils*
KLH (200 µg i.d.)	4	15	2 ml sera	7±0.7	82±10
1% SRBC+IFA	1	5	2 ml sera	1	7
CFA	1	5	4 ml sera	0	7
None	1	3	10% FCS	0	0
None	2	6	Nothing	0	9

*See legend to Table 1. IFA and CFA, incomplete and complete Freund's adjuvant.

immunity can depend solely on an accelerated development of reactions in the first skin tests of recipients. The serum transfer of basophil sensitivity to KLH reported here was validated by the absence of reactions to various irrelevant antigen skin tests in recipients of KLH immune sera, and also by the absence of macroscopic and microscopic delayed changes to KLH skin testing in non-immune animals and recipients of heterologous immune sera. Within the context of these controls, we conclude that serum transfer of CBH to KLH can be achieved.

P. W. ASKENASE
J. D. HAYNES
D. TAUBEN
R. DE BERNARDO

Department of Medicine,
Yale University School of Medicine,
333 Cedar Street, New Haven, Connecticut 06510

1 Dvorak, H. F., and Dvorak, A. M., *Prog. Immun. II*, 3, 171–181 (1974).
2 Askenase, P. W., *J. exp. Med.*, 138, 1144–1155 (1973).
3 Askenase, P. W., Haynes, J. D., and Hayden, B., *J. Allergy Clin. Immun.*, 53, 86 (1974).
4 Haynes, J. D., Kantor, F. S., and Askenase, P. W., *Fedn Proc.*, 34, 1039 (1975).
5 Askenase, P. W., and Hayden, B., *Fedn Proc.*, 34, 1039 (1975).
6 Jones, T. D., and Mote, J. R., *New Engl. J. Med.*, 210, 120–123 (1934).
7 Mote, J. R., and Jones, T. D., *J. Immun.*, 30, 149–167 (1936).
8 Catalona, W. J., Taylor, P. T., and Chretien, P. B., *Clin. exp. Immun.*, 12, 325–333 (1972).
9 Bloom, B. R., *New Engl. J. Med.*, 288, 908–909 (1973).
10 Turk, J. L., and Asherson, G. L., *Int. Archs Allergy appl. Immun.*, 21, 321–325 (1962).

Dependence of the contractile activation of skinned cardiac cells on the sarcomere length

FRANK[1] and Starling[2] have demonstrated that the systolic pressure developed by the heart decreased as its diastolic volume was altered in either direction from an optimum value. This law has been explained by variations of the number of cross bridges between the thin (actin) and the thick (myosin) filaments which generate force in individual cells[3,4] in accordance with the sliding filament theory[5].

Recent studies on skeletal muscle suggest that the release of Ca^{2+} from the sarcoplasmic reticulum (SR), which links the action potential of the surface membrane (sarcolemma) to the activation of the myofilaments, may also depend on the length of the cell. The activation of the myofilaments was found inhomogeneous at short sarcomere length (SL)[6] except when the fibre was treated with caffeine[7]. Furthermore, less Ca^{2+} was released at shorter length in fibres injected with the Ca^{2+}-bioluminescent protein aequorin[8,9]. The mechanism of the decrease in Ca^{2+} release, however, was not defined and no experiment was carried out on cardiac cells.

We have developed a simplified preparation to define the influence of SL on both the force developed by the myofilaments and the release of Ca^{2+} by the SR in cardiac cells. First, we have used single cells, thus eliminating the connective tissue which renders the relationship between the SL of each cell and the length of the preparation unpredictable[10]. Secondly, we have removed the sarcolemma of these cells by microdissection, thus placing the myofilaments and the SR in direct contact with solutions.

Cardiac tissue from the adult rat ventricle was homogenised into broken single cells (less than 60 µm in length and 15 µm in width) in a relaxing solution (composition given in Fig. 1). The remaining pieces of sarcolemma were removed from a broken cell by microdissection in a perfusion chamber placed on the stage of an inverted Reichert microscope[11]. The ends of the sarcolemma-free ('skinned') cell were impaled in a direction perpendicular to the axis of the myofibrils with two glass microtools. One microtool was immobilised and the other was connected to a highly sensitive photodiode isometric transducer[12]. The SL was measured on photomicrographs and on motion picture frames (100–400 f.p.s.) taken under differential interference microscopy (×160 objective with 1.30 numerical aperture) on at least 20 sarcomeres of each cell during both relaxation and contraction.

The concentration of Ca^{2+} ions (free $[Ca^{2+}]$) in the perfusing solutions was buffered with ethyleneglycol-*bis* (β aminoethyl ether) *N,N'*-tetraacetic acid (EGTA). We have previously demonstrated that two types of contractions can be obtained in this preparation[11]. The tonic contractions developed by the skinned cells when the free $[Ca^{2+}]$ is increased in the presence of a large buffer capacity (that is, a high total [EGTA]) correspond to the direct activation of

Fig. 1 Arrows indicate changes of free $[Ca^{2+}]$ or of SL. When one parameter was not modified the corresponding case was left unlabelled. A single cell was used to obtain the tracing of each row. The lengths of the cells were 45–55 µm when they were stretched to a 2.3 µm SL in relaxing solution. Their widths were: *a*, 12 µm; *b*, 11 µm; *c*, 11 µm; and *d*, 9 µm. SLs shorter than 1.5 µm were inferred from the variations of the distance between predetermined points of the cell. Striations caused by contraction bands, however, were visible at these short lengths. The total [EGTA] was 4×10^{-3} M in *a* and *b* and 5×10^{-5} M in *c* and *d*. All media contained 7 mM glucose and 18 mM Tris maleate, pH 7.0; temperature was maintained at 22 °C. The concentrations of Na_2EGTA, $CaCl_2$, Na_2ATP and $MgCl_2$ were varied to obtain the desired free $[Ca^{2+}]$, whereas the free $[Mg^{2+}]$ was held constant at 3.16×10^{-4} M and [MgATP] at 3.16×10^{-3} M. The ionic strength was kept constant at 0.16 M by appropriate addition of KCl. The following apparent association constants were used at pH 7.0: CaEGTA 4.9×10^6 M^{-1}, MgEGTA 40 M^{-1}, CaATP 5×10^3 M^{-1}, MgATP 11.4×10^3 M^{-1}. The amplitude of the tonic contractions was maximum for 10^{-5} M free Ca^{2+} and zero for a free $[Ca^{2+}]$ smaller than 2×10^{-7} M in the presence of 4.0 mM total EGTA. In contrast, phasic contractions could be triggered by a free $[Ca^{2+}]$ as low as 4×10^{-8} M in the presence of 5×10^{-5} M total EGTA. A suprathreshold free $[Ca^{2+}]$ (5×10^{-8} M) was used to trigger the phasic contractions in this study.
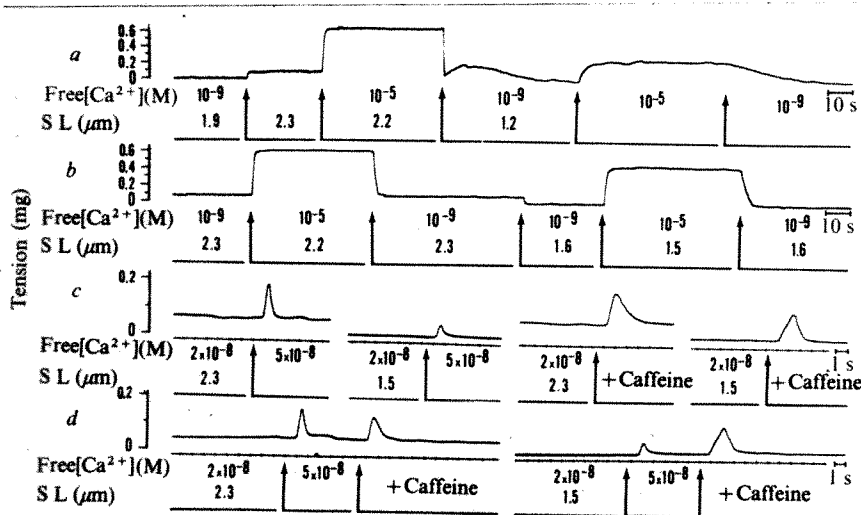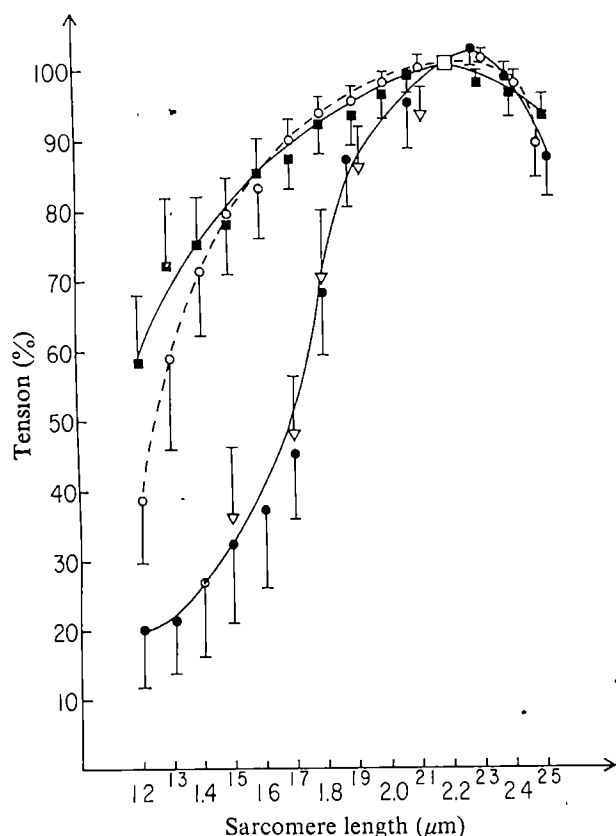
Fig. 2 Effects of the variations in SL (measured during contraction) on tonic contractions induced by direct activation of myofilaments (■) and on the phasic contractions induced by a release of Ca^{2+} from the SR triggered either by Ca^{2+} (●, △) or by caffeine (○). Tensions (plateau of tension for the tonic contractions or peak tension for the phasic contractions) were measured as percentages of tension developed by the same type of contraction at a 2.2 μm SL_x(□). Each point represents the mean of 12–15 measurements ,and each vertical bar represents 1 s.d. (shown in only one direction for clarity). Lengths of the cells were 41–63 μm when they were stretched to a 2.3 μm SL in relaxing solution. Their widths were 10±2 μm except for △, for which the widths of the cells were 5±1 μm.

the myofilaments by the Ca^{2+} present in the buffer (Fig. 1*a* and *b*). In contrast, the phasic contractions triggered by a small variation of the free $[Ca^{2+}]$ in the presence of a slight EGTA buffering correspond to a Ca^{2+}-triggered release of Ca^{2+} from the SR (Fig. 1*c* and *d*). By this mechanism, a small trans-sarcolemmal influx of Ca^{2+} into the intact cell would trigger a release of Ca^{2+} from the SR sufficient to activate the myofilaments[11].

The effects of SL on the tonic contractions by direct activation of the myofilaments were studied in the presence of 4×10^{-3} M total EGTA (Fig 1*a* and *b*). The slack SL in relaxing solution was 1.9 μm (Fig. 1*a*). A larger SL (2.3 μm) was obtained by stretching the cell, which resulted in the development of a resting tension. A large active tension was induced by full activation with 10^{-5} M free Ca^{2+}. To obtain SLs much shorter than the slack SL, the cell was released by rapidly moving the end not attached to the transducer, and a relaxing solution was applied (Fig. 1*a*). A rapid relaxation followed by a slow redevelopment of tension and a slow relaxation were observed without change of length and without variation in the spacing of the contraction bands observed at this short SL. When the relaxation was completed the contracting solution was applied, and a plateau of tension was slowly reached with no significant variation in SL (<0.5 μm). To obtain SLs moderately shorter than the slack SL, one end of the cell was simply compressed during relaxation (Fig. 1*b*) causing a

waving of the myofibrils, which became straight during contraction.

The compliance of the transducer (2–3 μm mg^{-1}) and the passive lengthening of damaged tissue in the areas of attachment to the microtools enabled a significant shortening of the SL to occur during contraction. Accordingly, the active tension was expressed as a function of the SL measured during the contraction rather than during the relaxation (Fig. 2). The maximum tonic tension was observed at a 2.2 μm SL and was defined as 100% tension in each cell. The tension obtained at other SLs was expressed as a percentage of this maximum value. Tensions of 58±10% (s.d.) at a 1.2 μm SL and of 78±7% at a 1.5 μm SL were obtained. These results are similar to those observed on skinned fibres of skeletal muscle[13] and are very different from those observed in the intact cardiac muscle where no active tension is developed at a length 30% below optimum[3,4]. Therefore, the Frank–Starling law[1,2] cannot be explained entirely by a dependence of the maximum number of cross bridges between the thin and the thick filaments on the SL.

The effects of SL on the phasic contractions induced by Ca^{2+} released from the SR were studied in the presence of 5×10^{-5} M total EGTA (Fig. 1*c* and *d*). Short SLs were obtained by the same procedures as for the tonic contractions but with a relaxing solution containing 5×10^{-5} M total EGTA and 2×10^{-8} M free Ca^{2+}. The amplitude of these contractions decreased rapidly when the SL was decreased. It was maximum at a 2.3 μm SL (102±3% of the tension developed at a 2.2 μm SL which was chosen arbitrarily as reference) and only 32±11% at a 1.5 μm SL (Figs 1*c* and 2). The activation of the cell was homogeneous at a 1.5 μm SL, as demonstrated by the absence of wavy myofibrils during contraction. Furthermore, no significant differences were observed in the results obtained in preparations of various widths (5±1 μm compared with 10±2 μm; Fig. 2). These observations suggest that the Ca^{2+} present in the buffer diffused homogeneously throughout the cells.

Phasic contractions were also triggered by 5×10^{-3} M caffeine (Fig. 1*c*), which releases a large fraction of the Ca^{2+} contained in the SR (ref. 14). When the SL was decreased from 2.2 to 1.4 μm the percentage decrease in amplitude of these caffeine-induced contractions was not significantly different from that of the tonic contractions elicited by the direct activation of the myofilaments (Fig. 2). This suggests that the amount of Ca^{2+} released by caffeine and the Ca^{2+} content of the SR (by inference) are not dependent on the SL in this range of SLs. A contraction was induced by 5×10^{-3} M caffeine at a fixed interval after the contraction induced by Ca^{2+}-triggered release of Ca^{2+} (Fig. 1*d*). By this method[15], the amount of Ca^{2+} remaining in the SR after the Ca^{2+}-triggered release can be unmasked. The contraction induced by caffeine at a 1.5 μm SL (after the small Ca^{2+}-triggered contraction) was larger than that induced at a 2.3 μm SL (after the large Ca^{2+}-triggered contraction) Thus, a decrease in SL would not decrease the Ca^{2+} content of the SR but would decrease the amount of Ca^{2+} released by the Ca^{2+}-triggered process. Furthermore, the slow rate of tension development and the relatively rapid relaxation, which were consistently observed in the contractions induced by caffeine at short SLs, may be related to a decrease in rate of Ca^{2+} release with no modification or even an increase in rate of Ca^{2+} sequestration by the SR.

The amplitude of tonic and phasic contractions decreased when the SL was increased above 2.2 or 2.3 μm (Fig. 2), in agreement with the sliding-filament theory[5]. The apparent discrepancy between this finding and that reported in partially activated skinned fibres of skeletal muscle[16] will not be discussed because SLs larger than 2.5 μm were not used in the data reported here.

In conclusion, these results suggest that decreasing the SL

below optimum results in a partial inhibition of the process of Ca^{2+}-triggered release of Ca^{2+} from the SR of cardiac cells. This mechanism may contribute to the explanation of the Frank–Starling law of the heart[1,2].

A.F. is an Established Investigator of the American Heart Association.

ALEXANDRE FABIATO
FRANÇOISE FABIATO

Cardiovascular Research Laboratory,
Harvard Medical School and Peter Bent Brigham Hospital,
721 Huntington Avenue,
Boston, Massachusetts 02115

1 Frank, O., Z. *Biol.*, 32, 370–447 (1895).
2 Starling, E. H., *Linacre Lecture on the Law of the Heart* (Longmans, London, 1918).
3 Sonnenblick, E. H., Spiro, D., and Cotrell, T. S , *Proc. natn. Acad. Sci. U.S.A.*, 49, 193–200 (1963).
4 Jewell, B. R., and Noble, M. I. M. (ed.) *The Physiological Basis of Starling's Law of the Heart*, Ciba Foundation symposium, 24 (new series), (Elsevier, Amsterdam, 1974).
5 Gordon, A. M., Huxley, A. F., and Julian, F. J., *J. Physiol , Lond*, 184, 170–192 (1966).
6 Taylor, S. R., and Rudel, R., *Science*, 167, 882–884 (1970).
7 Rudel, R., and Taylor, S. R., *Science*, 172, 387–388 (1971).
8 Blinks, J. R., *Fedn Proc.* (in the press).
9 Taylor, S. R , in *The Physiological Basis of Starling's Law of the Heart*, Ciba Foundation symposium, 24 (new series), (edit. by Jewell, B. R., and Noble, M. I. M.), 93–109 (Elsevier, Amsterdam, 1974).
10 Winegrad, S., *J. gen. Physiol.*, 64, 343–355 (1974)
11 Fabiato, A., and Fabiato, F., *Eur. J. Cardiol.*, 1, 143–155 (1973); *Nature*, 253, 556–558 (1975); *J. Physiol., Lond.* (in the press).
12 Meiss, R. A., *J. appl. Physiol.*, 37, 459–463 (1974).
13 Schoenberg, M., and Podolsky, R. J., *Science*, 176, 52–54 (1972).
14 Weber, A., and Herz, R., *J. gen. Physiol.*, 52, 750–759 (1968).
15 Endo, M., Tanaka, M., and Ogawa, Y., *Nature*, 228, 34–36 (1970).
16 Endo, M., *Nature new Biol.*, 237, 211–213 (1972).

Morphine antagonises prostaglandin E₁- mediated inhibition of human platelet aggregation

COLLIER and Roy[1] have proposed that in morphine-sensitive neurones the receptor site may be adenyl cyclase, which is physiologically stimulated by prostaglandin E_1 (PGE_1) and pharmacologically blocked by opiates. An hypothesis has been put forward that morphine exerts its analgesic effect by inhibiting the stimulation by PGE_1 of cyclic AMP formation in appropriate neurones[2], whereas acute side effects of opiates may be caused by stimulation of prostaglandin biosynthesis[3].

We wondered if a similar receptor site for PGE_1 and morphine exists in blood platelets. The basis for this assumption is that inhibition of platelet aggregation by PGE_1 (refs 4 and 5) and PGE_2 (ref. 5) is associated with activation of adenyl cyclase and accumulation of cyclic AMP in platelets. Therefore the final effect of PGE on platelets (that is, aggregation) is mediated through the same biochemical pathway as some central effects of PGE (for example, pain sensation). Moreover platelets have the enzymic system that synthesises prostaglandins[6,7].

Platelet aggregation was studied by the turbidometric technique of Born[8]. Methods for blood collection of citrate platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were as described by Han and Ardlie[9]. PPP was used to adjust the platelet count of PRP to 250,000 μl^{-1}. The aggregating agents used were ADP (Sigma) at a final concentration of 2 μM and DL(±)-adrenaline bitartarate (Sigma) at a final concentration of 50 μM.

Preincubation of PRP with PGE_1 (Upjohn) at a final concentration in the range 0.028–0.565 μM inhibited adrenaline-induced aggregation in a dose-dependent manner. PGE_1 was added to PRP 30 s before adrenaline. Then PGE_1 flattened the slope of the first phase and delayed the appearance of the second phase of aggregation. A short

(30-s) preincubation of PRP with morphine hydrochloride (Polfa) at concentrations in the range 1.33–26.6 μM reduced or abolished the protective action of PGE_1 against adrenaline-induced aggregation (Fig. 1). In some platelet preparations PGE_1 caused a complete disappearance of the second phase of aggregation. In these preparations, preincubation with morphine restored the aggregating power of adrenaline. Usually the optimal active concentration of morphine was in the range 1 33–13.3 μM. Higher concentrations than the latter often produced a paradoxical reappearance, or even enhancement of PGE_1-dependent inhibition of adrenaline-induced platelet aggregation (Fig. 1). Some experiments with levorphanol hydrochloride (Dromoran, Roche) have shown that 5–20 times lower concentrations of the drug were needed to reach an effect equivalent to that of morphine. Incubation of PRP with morphine (26.6 μM) for up to 5 min neither changed the turbidity of PRP nor influenced markedly adrenaline-induced aggregation. In case of a distinct biphasic curve of the adrenaline-induced aggregation, morphine (2.66–26.6 μM) either shortened or erased the intercept between both phases.

The patterns of inhibition by PGE_1 of ADP-induced aggregation were somewhat different from those observed for adrenaline. Thus, PGE_1 not only reduced the rate and intensity of aggregation but also gave rise to a delay between the addition of ADP and the onset of aggregation. All these PGE_1-mediated protective effects against ADP-induced aggregation were reduced or abolished by a preincubation of PRP with morphine (Fig. 2). In the absence of PGE_1, morphine (26.6 μM) did not influence ADP-induced aggregation.

The ability of PGE_1 to inhibit aggregation varied greatly in different platelet donors. Similarly, the potency of the antagonistic action of morphine against PGE_1-mediated

Fig. 1 Prevention by morphine of the anti-aggregating effects of PGE_1 in adrenaline-induced aggregation of human PRP. For the control (*a*) 0.1 ml adrenaline bitartarate solution was added to 1.7 ml PRP and 0.2 ml distilled water, to yield a final concentration of 50 μM of adrenaline. To show the anti-aggregating effect of PGE_1 (*d*), 30 s before adrenaline was added, 0.1 ml sodium salt of PGE_1 was instilled into 1.7 ml PRP and 0.1 ml distilled water. The final concentration of PGE_1 was 0.028 μM. The antagonism between morphine and PGE_1 is shown in *b* and *c*. Morphine hydrochloride in a volume of 0.1 ml was added to 1.7 ml PRP 30 s before PGE_1, and 60 s before adrenaline. The final concentrations of morphine were 2.66 μM (*b*) and 26.6 μM (*c*). With a rise in morphine concentration the diminution of its preventive action was observed. Morphine at a concentration of 133 μM (not shown) actually enhanced the anti-aggregating effect of PGE_1.
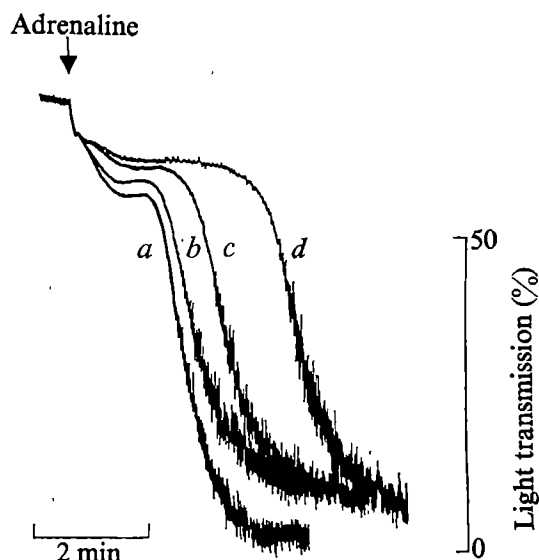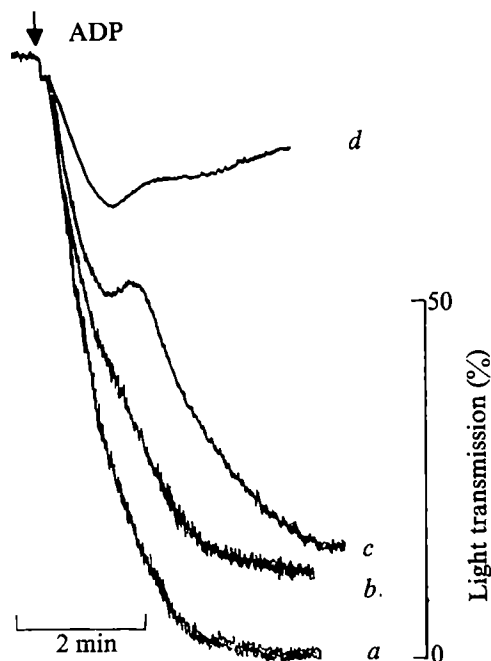
Fig. 2 Prevention by morphine of the anti-aggregating effects of PGE_1 in ADP-induced aggregation of PRP. The procedure was similar to that described in Fig. 1. Instead of adrenaline, ADP was used at a final concentration of 2 µM. PGE_1 was used at a final concentration of 0.14 µM. *a*, ADP; *b*, morphine (26.6 µM) $+PGE_1+ADP$; *c*, morphine (2.66 µM)$+PGE_1+ADP$; *d*, PGE_1 $+ADP$. A rise in morphine concentration enhanced the preventive action of morphine against the anti-aggregating effect of PGE_1.

protection of adrenaline-induced or ADP-induced aggregation differed from one plasma to another.

Summing up, the antagonism between PGE_1 and morphine similar to that described in rat brain homogenates[1,2] may also be demonstrated in human platelets. It is probable that adenyl cyclase is the common target for PGE_1 and morphine in platelets as it is in neurones[2]. One of the common side effects of opiates is histaminaemia[10]. Basophils[11] and mast cells, like platelets[4,5], have adenyl cyclase which is stimulated by PGE_1. Because of this, PGE_1 inhibits histamine release from basophils[11] and mast cells[12]. Perhaps morphine blocks this PGE_1-mediated, protective mechanism and therefore histaminaemia appears.

It may be possible to use the preventive effects of opiates against the anti-aggregating action of PGE_1 for *in vitro* screening of potency of narcotic analgesics, as well as for the study on the character of opiate receptors[13].

RYSZARD J. GRYGLEWSKI
ANDRZEJ SZCZEKLIK
KRZYSZTOF BIERON

*Departments of Pharmacology and Allergy,
and Clinical Immunology,
Copernicus Academy of Medicine in Cracow,
31–531 Cracow, 16 Grzegórzecka, Poland*

1 Collier, H. O. J., and Roy, A. C., *Nature*, 248, 24–27 (1974).
2 Collier, H. O. J., and Roy, A. C., *Prostaglandins*, 7, 361–376 (1974).
3 Collier, H. O. J., McDonald-Gibson, W. J., and Saeed, S. A., *Nature*, 252, 56–58 (1974).
4 Shio, H., Shaw, J., and Ramwell, P., *Ann. N.Y. Acad. Sci.*, 185, 327–335 (1971).
5 Bruno, J. J., Taylor, L. A., and Droller, M. J., *Nature*, 251, 721–723 (1974).
6 Srivastava, K. C., and Clausen, J., *Lipids*, 7, 762–765 (1972).
7 Hamberg, M., and Samuelsson, B., *Proc. natn. Acad. Sci. U.S.A.*, 71, 3400–3405 (1974).
8 Born, G. V. R., *Nature*, 194, 927–929 (1962).
9 Han, P., and Ardlie, N G., *Br. J. Haemat.*, 26, 373–389 (1974).
10 Feldberg, W., and Paton, W. D M., *J. Physiol., Lond.*, 114, 490–509 (1951).
11 Lichtenstein, L. M., in *Asthma* (edit. by Austen, K. F., and Lichtenstein, L. M.), 91–103 (Academic, New York and London, 1973).
12 Albro, P., Thomas, R., and Fishbein, L., *Prostaglandins*, 1, 133 (1972).
13 Goldstein, A., *Life Sci.*, 14, 615–623 (1974).

Morphine elevates levels of cyclic GMP in a neuroblastoma X glioma hybrid cell line

THE use of cell lines derived from tumours of the nervous system as models for both neurones and glia has become well established. Clonal lines derived from mouse neuroblastoma C1300 have been shown to possess many properties characteristic of neurones[1]. Several such properties are more strongly expressed in hybrids between mouse neuroblastoma and rat glioma cells[2-4]. They contain choline acetyltransferase[2-4], clear and dense core vesicles[5] and dopamine-β-hydroxylase[4]. In the presence of prostaglandin E_1 (PGE_1) the hybrid cells strongly increase their intracellular levels of cyclic AMP^6. The effect of PGE_1 is antagonised by noradrenaline[7], acetylcholine[8] and morphine[9-11]. The latter finding is in agreement with that in brain homogenates[12]. In addition, morphine receptors could be detected in the hybrid cells[13].

Fig. 1 Stimulation by morphine of the levels of cyclic GMP in the hybrid line 108CC15. Cells were grown in plastic dishes (Greiner, 8.5 cm diameter)[6,15]. One hour before the experimental incubations in the presence of morphine, the growth medium was replaced by the incubation medium[15]. Morphine was added as described[10] and the incubation ended 10 min later with the removal of the medium and addition of 3 ml ice-cold 8% (w/v) trichloroacetic acid. For control of recoveries, tritiated cyclic AMP and cyclic GMP were added. For removal of the acid, the solution was extracted 5 times with ether. The cyclic nucleotides were separated on a column of BioRad AG 1×2 (Cl$^{-200-400}$ mesh, 4×0.5 cm). After application of the sample, the column was washed with 10 ml H_2O and eluted with 6 ml 0.05 mol l^{-1} HCl (cyclic AMP) and subsequently another 6 ml 0.5 mol l^{-1} HCl (cyclic GMP). After neutralisation with 3 mol l^{-1} Tris base, the cyclic GMP fraction was rerun on regenerated columns, to remove traces of cyclic AMP. After lyophilisation, the eluates were analysed for cyclic AMP and cyclic GMP. The results are mean values ±s.d. of data obtained from 4 parallel plates. Passage number 15; 1.2×10^6 viable cells per plate; viability 92%. ●, cyclic GMP; ○, cyclic AMP.
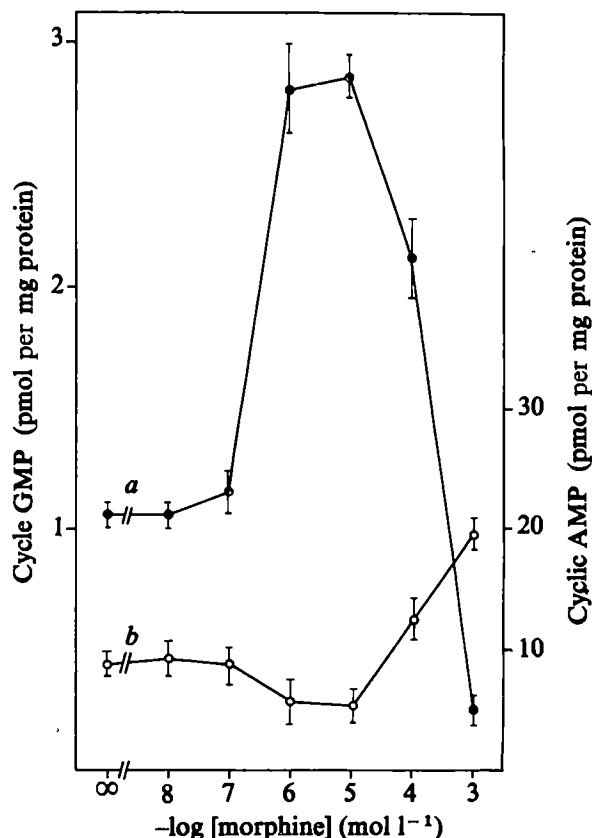
In view of the opposing effects of cyclic AMP and cyclic GMP[14], we studied the levels of cyclic GMP in the hybrid cells in response to morphine. After incubation (10 min) in the presence of the drugs under study, the cyclic nucleotides were extracted from the cells by trichloroacetic acid and separated by anion exchange chromatography. Cyclic AMP[15,16] and cyclic GMP (P. Wunderwald, D. van Calker, R. G., J. T. and B. H., unpublished) were determined by protein binding assays.

It can be seen in Fig. 1 that morphine (0.1 to 10 μmol l^{-1}) elevates cyclic GMP levels and that there seems to be a concomitant depression in basal levels of cyclic AMP. At higher morphine concentrations (0.1 to 1 mmol l^{-1}) this elevation is reversed to cyclic GMP values below those found in the absence of morphine and accompanying this there is a rise in cyclic AMP levels. The intracellular concentrations of cyclic GMP are about one order of magnitude lower than those of cyclic AMP and the absolute changes are much smaller for cyclic GMP than for cyclic AMP.

Naloxone, a potent antagonist of the action of morphine[17] inhibits the elevation of cyclic GMP levels produced by morphine (Fig. 2). With naloxone concentrations two orders of magnitude lower than those of the morphine used as stimulant, levels of cyclic GMP are reduced below basal (Fig. 2). In the absence of morphine, naloxone decreases cyclic GMP and increases cyclic AMP (Fig. 2) levels, but such changes occur at concentrations (0.1 to 1.0 mmol l^{-1}) much higher than those necessary to reduce cyclic GMP levels elevated by morphine.

The elevation by morphine of cyclic GMP levels could be mimicked by levorphanol, a congener of morphine, but not by its inactive enantiomer dextrorphan (Fig. 3). Cyclic GMP levels were increased at levorphanol concentrations of 0.1 to 10 μmol l^{-1}. An inhibition of this increase occurred at higher concentrations (Fig. 3). In response to levorphanol, the level of cyclic AMP changes inversely to that of cyclic GMP (Fig. 3). At high concentrations, dextrorphan reduces the basal level of cyclic GMP, while

Fig. 2 Naloxone inhibits the elevation of levels of cyclic GMP caused by morphine. 1.4×10^6 viable cells per plate (passage number 17, viability 90%) were incubated at varying concentrations of naloxone in the presence (●) and absence (■; ○) of morphine (10 μmol l^{-1}). ● and ■, cyclic GMP; ○, cyclic AMP.
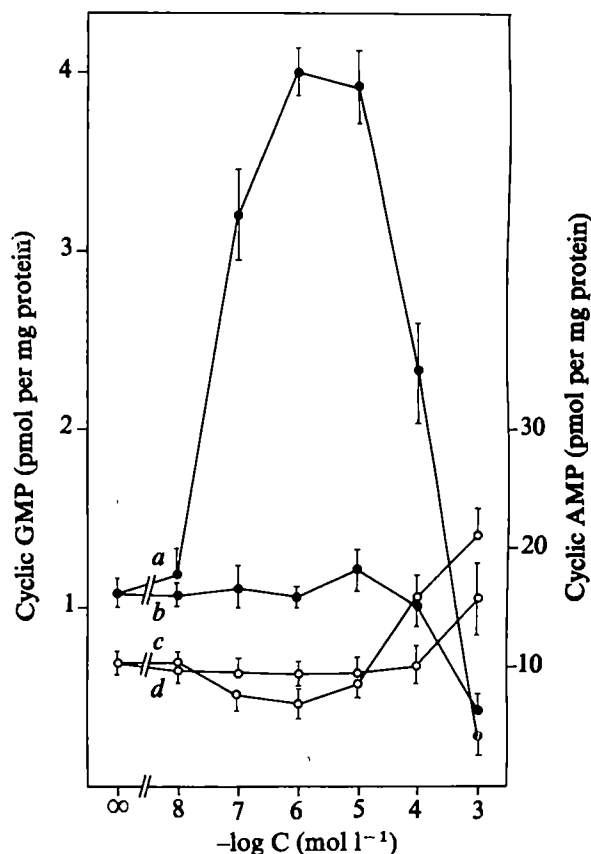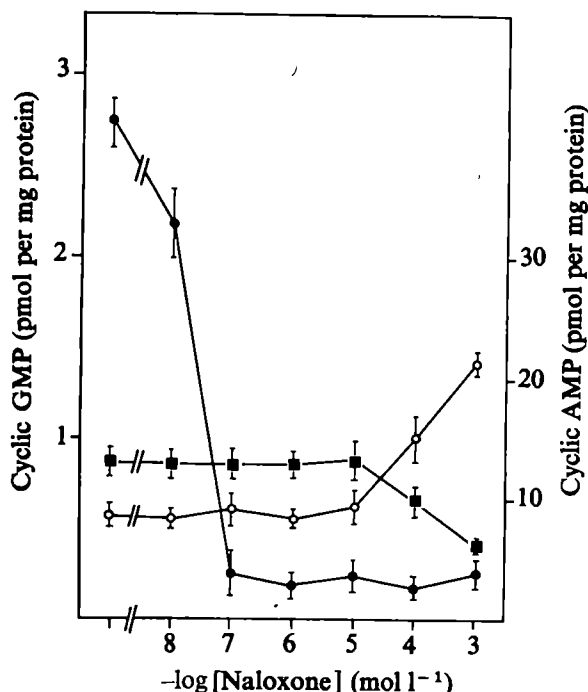
Fig. 3 Effect of levorphanol and dextrorphan on levels of cyclic GMP and cyclic AMP. Cells were incubated with levorphanol (curves a and c) or dextrorphan (curves b and d). Curves a and b, cyclic GMP; curves c and d, cyclic AMP. Passage number 12; 1.2×10^6 viable cells per plate, viability 91%. The level of cyclic GMP in the presence of 10 μmol l^{-1} morphine was 2.9 ± 0.2 pmol mg^{-1} protein.

it elevates the level of cyclic AMP (Fig. 3). In two ways the elevation by morphine of levels of cyclic GMP in the hybrid line was demonstrated to be a specific opiate action. It was antagonised by naloxone, and mimicked by levorphanol but not dextrorphan. An analogous specificity was found for the inhibition by opiates of the elevation of cyclic AMP levels by PGE$_1$[7,10]. The doses of morphine required for a 50% increase of the cyclic GMP level (Fig. 1) and a 50% depression of the cyclic AMP level[10] agree within one order of magnitude. This similarity could suggest that the depressing effect of morphine on cyclic AMP levels[7,9,10] is perhaps mediated by cyclic GMP. In the hybrid cells noradrenaline and acetylcholine, like morphine, lower cyclic AMP levels[7-10] and increase levels of cyclic GMP (R. G. and B. H., unpublished). The negative regulatory influence of cyclic GMP on cyclic AMP levels seems to be a more general phenomenon. But, the reverse hierarchy of the cyclic nucleotides is also found; at increased concentrations, morphine or naloxone cause a rise in the level of cyclic AMP whereas the cyclic GMP level is depressed (Figs 1 and 2). What is the molecular basis of these antagonisms? Most of the actions of cyclic nucleotides are mediated through the agency of protein kinase and phosphorylation reactions. Thus, it is interesting to note that such phosphorylation reactions have been shown to inhibit adenyl cyclase activities[18].

Besides antagonising the effect of morphine at concentrations below 0.1 μmol l^{-1} and increasing levels of cyclic AMP at concentrations above 10 μmol l^{-1}, naloxone causes a third effect. Although, alone at concentrations less than 10 μmol l^{-1} it does not depress the level of cyclic AMP below the control value, it does so in the presence of

10 μmol l^{-1} morphine (Fig. 2): that is, it overcompensates the stimulatory effect of morphine. Experiments are under way in an attempt to elucidate the mechanism of this unusual action.

The opposite actions of morphine or levorphanol at low and high concentrations suggest that these opiate effects are mediated by receptors of high and low opiate affinity, respectively. Since, at high concentrations levorphanol and dextrorphan have the same effects, the low affinity receptor should lack stereospecificity. High and low affinity opiate binding sites of high and low stereospecificity, respectively, have been detected in brain[19]. Opiates do not normally occur in animals. There should therefore exist in brain at least two different natural ligands of hormone-like character, which bind to the "opiate receptors" and which are mimicked probably by different parts of the opiate molecules. We like to suggest that the actions of these endogenous ligands should be mediated by cyclic GMP and cyclic AMP, respectively. We are trying to isolate and identify these substances.

We cannot yet decide whether in opiate action the increase in levels of cyclic GMP or the depression of (hormonally increased?) cyclic AMP levels by elevated levels of cyclic GMP is the key event. This is an important issue, since it has been proposed[12] that a mechanism, whereby morphine-like drugs exert their analgesic and other effects, is the inhibition of the stimulation by PGE₁ of cyclic AMP production. But morphine also inhibits the stimulation of cyclic AMP formation caused by other agents[7]. Thus, the antagonisms between PGE₁ and morphine may or may not be a fortuitous combination of little physiological significance.

ROBERT GULLIS
JÖRG TRABER
BERND HAMPRECHT

Max-Planck-Institut für Biochemie,
8033 Martinsried,
Federal Republic of Germany

1 Sato, G., in *Current Topics in Neurobiology*, 1 *Tissue Culture of the Nervous system*, (Plenum Press, New York and London, 1973).
2 Amano, T., Hamprecht, B., and Kemper, W., *Expl Cell Res.*, 85, 399–408 (1974).
3 Hamprecht, B., *Hoppe-Seyler's Z. physiol. Chem.* 355, 109–110 (1974).
4 Hamprecht, B., in 25 *Colloquium der Gesellschaft fur Biologische Chemie* (edit. by Jaenicke, L.), 391–423 (Springer, Berlin, 1974)
5 Daniels, M. P., and Hamprecht, B., *J. Cell Biol.*, 63, 691–699 (1974).
6 Hamprecht, B., and Schultz, J., *Hoppe-Seyler's Z. physiol. Chem.*, 354, 1633–1641 (1973).
7 Traber, J., Reiser, G., Fischer, K., and Hamprecht, B., *FEBS Lett.*, 52, 327–332 (1975).
8 Traber, J., Fischer, K., Buchen, C., and Hamprecht, B., *Nature*, (in the press).
9 Traber, J., thesis, Univ. Munich (1973).
10 Traber, J., Fischer, K., Latzin, S., and Hamprecht, B., *Nature*, 253, 120–122 (1975).
11 Sharma, S. K., Nirenberg, M., and Klee, W. A., *Proc natn. Acad. Sci. U.S.A.*, 72, 590–594 (1975).
12 Collier, H. O. S., and Roy, A. C., *Nature*, 248, 24–27 (1974).
13 Klee, W. A., and Nirenberg, M., *Proc. natn. Acad. Sci U.S.A.*, 71, 3474–3477 (1974).
14 Goldberg, N. D., Haddox, M. K., Hartle, D. K., and Hadden, J. W., *Proc. fifth Int. Cong. Pharmacol.*, 5, 146–169 (1973).
15 Traber, J., Fischer, K., Latzin, S., and Hamprecht, B, *Proc. ninth Int. Cong. Colloqium Internationale Neuropsychopharmacologicum* (in the press).
16 Gilman, A. G., *Proc. natn. Acad. Sci. U.S A.*, 67, 305–312 (1970).
17 Martin, W. R., *Pharmac. Rev.*, 19, 463–521 (1967).
18 Constantopoulos, A., and Najjar, V. A., *Biochem. biophys. Res. Commun.*, 53, 794–799 (1973).
19 Goldstein, A., Lowney, L. I., and Pal, B., *Proc. natn. Acad. Sci U.S.A.*, 68, 1742–1747 (1971).

2-Hydroxy-oestradiol-17β as a possible link in steroid brain interaction

IN spite of wide evidence for their existence, mechanisms of steroid–brain interaction are poorly understood. It has been reported that hydroxylation of oestrogens occurs in the liver[1,2] and that hydroxylated oestrogens such as 2-hydroxy-oestradiol-17β (2-OHE₂) are strong competitive

inhibitors of the methylation of catecholamines by catechol-O-methyltransferase[3]. We report for the first time the action of 2-OHE₂ on the brain. Our experiments demonstrate that 2-OHE₂ lowered plasma levels of the anterior pituitary luteinising hormone (LH). A response was recorded from the amygdala, which accumulate gonadal steroids[4,5] and participate in the regulation of the anterior pituitary.

Nine adult miniature pigs (Göttingen strain) were orchidectomised at least 4 weeks before the experiment, and kept in daylight and otherwise standard conditions. Stainless steel tubing (22 G) with inner exchangeable tubing (27 G) was stereotaxically and bilaterally implanted into the amygdala Histology showed that in seven animals the tip of the implant was located in the basolateral and cortico-medial amygdala and in two animals outside the amygdala. Two weeks after the implantation, a catheter was placed into the vena jugularis externa for the collection of blood samples. Treatment started 5 d later. Each individual unanaesthetised animal was microinjected by way of the inner cannula using a 10 μl Hamilton syringe (2 μl single injection; stock was made up from 7 ml 0.9% NaCl and 3 ml 20% ethanol (EtOH)). Several days later 2-OHE₂ (2 μl NaCl-EtOH containing very freshly dissolved 60 ng 2-OHE₂) was microinjected. This was followed by another NaCl-EtOH control treatment. Microinjections lasted no longer than 1–2 min. Blood samples (2 ml) were collected at 15 min intervals from each animal: nine samples 120–0 min before, eight 15–120 min and eight 135–240 min after the microinjection. At 24 and 48 h after injection, blood was collected again at 15 min intervals for 90 min. A 4-day recovery was allowed between treatments. Plasma LH was analysed by double-antibody radioimmunoassay[6]. In addition to the two control animals with implants just outside the amygdala, elaborate experiments were performed to exclude leakage to other brain regions, the pituitary or the peripheral circulation. These included application of radioactive steroids into rat and pig brains, estimations of the distance of diffusion, as well as radioimmunological determination of circulating steroid levels after steroid microinjections identical to those of 2-OHE₂ (N.P. and F.E., unpublished).

Table 1 shows that 2 μl NaCl-EtOH had no effect on plasma LH levels. 2-OHE₂ decreased plasma LH significantly at 13 out of 27 points of time. The number of animals with a significant decrease in plasma LH after injection increased with time. Microinjection of any substance outside the amygdala did not alter plasma LH significantly. An individual response during the entire experiment is shown in Fig. 1. The consistent changes in plasma LH after the microinjection of 2-OHE₂ into the amygdala clearly show that this compound can alter brain pituitary function. The well known fact that oestrogens concentrate in the amygdala[4,5] and observation that increased catecholamine content in the amygdala is associated with lowered plasma LH levels[7] are consistent with the mechanisms of action of

Table 1   Effect of microinjections of 2-hydroxy-oestradiol-17β (2-OHE₂) on plasma LH levels in orchidectomised adult miniature pigs

Time after microinjection (h)	NaCl-EtOH*		2-OHE₂ (60 ng–2 μl NaCl-EtOH)			NaCl-EtOH	
	n	NC	n	D	NC	n	NC
0–2	7	7	7	2	5	5	5
2–4	7	7	7	3	4	5	5
24–25.5	7	7	7	4	3	4	4
48–49.5	7	7	6	4	2	2	2

* 2 μl NaCl-EtOH (7 ml 0.9% NaCl + 3 ml 20% ethanol).
n, Number of animals. Two animals in which the tips of cannulas were outside the amygdala are not considered in the Table. They did not cause a change.
D, Decrease in plasma LH levels. Significant (at least $P < 0.05$, Student's t test) when compared with the control periods.
NC, no change.
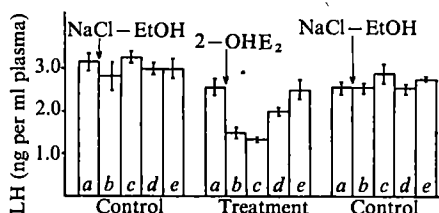
Fig. 1 Plasma LH (mean±s.e.m.; LER-786-3) after micro-injection of 2-OHE₂ into the amygdala of an individual orchid-ectomised adult miniature pig. NaCl–EtOH: 2 μl of a stock made from 7 ml 0.9% NaCl and 3 ml 20% EtOH; 2-OHE₂: 2 μl NaCl–EtOH containing 60 ng OHE₂; number of samples per block: seven to nine, obtained at 15 min intervals. Each column (*b–e*) was compared with the corresponding control period (*a*) by Student's *t*- test. 2-OHE₂ treatment resulted in significant decrease in columns (*b*) and (*c*), ($P \leq 0.001$) and in column (*d*), ($P \leq 0.01$). *a*, 0–2 h before microinjection; *b*, 0–2 h after microinjection; *c*, 2–4 h after microinjection; *d*, 24–25.5 h after microinjection, *e*, 48–49.5 h after microinjection.

2-OHE₂ (ref. 3). It remains to be established whether it is crossing the blood–brain barrier or is synthesised in the brain, or both. It is also necessary to determine whether 2-OHE₂ actions occur in other oestrogen-concentrating brain areas.

2-Hydroxy-oestradiol-17β was kindly provided by Dr H. Breuer, Bonn, Germany, the LH antiserum by Dr G. D. Niswender, Fort Collins, USA and the LH standard by Dr L. E. Reichert, Atlanta, USA. This work was supported by the Deutsche Forschungsgemeinschaft.

NAHID PARVIZI
FRANZ ELLENDORFF

Section for Reproductive Biology,
Department of Animal Science,
3400 Göttingen,
Albrecht-Thaer-Weg 1, FRG

1 Breuer, H., Vogel, W., and Knuppen, R., *Z. physiol. Chem.*, **327**, 217–224 (1962).
2 Knuppen, R., Lubrich, W., Haupt, O., Ammerlahn, N., and Breuer, H., *Z. physiol. Chem.*, **350**, 1067–1075 (1969).
3 Fishman, J., Bradlow, H. L., Gallagher, T. F., *J. biol. Chem.*, **235**, 3104–3107 (1960).
Fishman, J., Hellman, H. L., Zumoff, B., and Gallagher, T. F., *J. clin. Endocrinol.*, **25**, 365–367 (1965).
4 Pfaff, D. W., *Science*, **161**, 1355–1356 (1968).
5 Stumpf, W. E., *Science*, **162**, 1001–1003 (1968).
6 Pomerantz, D. K., Ellendorff, F., Elsaesser, F., Konig, A., and Smidt, D., *Endocrinology*, **94**, 330–335 (1974).
7 Lichtensteiger, W., and Keller, P. J., *Brain Res.*, **74**, 279–303 (1974).

Amino acid synthesis through biogenetic-type CO₂ fixation

In bacterial photosynthesis, reductive CO₂ fixation was thought to proceed through activation of carboxylic acid by coenzyme A followed by the reductive incorporation of CO₂ in the presence of reduced ferredoxin (a kind of non-haem iron–sulphur protein) and an enzyme, pyruvate synthase, as shown in equation (1). The reaction takes place *in vitro* in the same way using CoA, reduced ferredoxin and pyruvate synthase[1].

$$CH_3C{\sim}\textcircled{P} \longrightarrow CH_3C\text{-S-CoA} \xrightarrow[\substack{ferredoxin_{red.} \\ pyruvate\ synthase}]{CO_2} CH_3CCO_2H$$

(1)
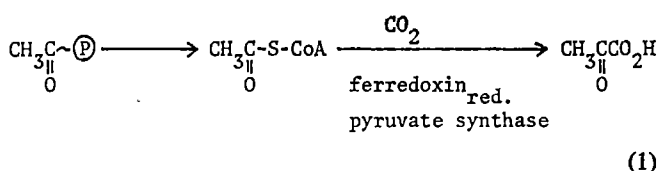
During attempts to prepare some artificial photosynthetic models, we have discovered a similar biogenetic-type reaction which uses a simple catalyst modelling ferredoxin activity. As catalysts, we used some known iron–sulphur

complexes such as Schrauzer's complex[2], Yang's complex[3], and Holm's complex[4].

We now wish to report that completely artificial catalytic systems, one of which consisted of a simple alkyl mercaptan, one of the ferredoxin models, and an inorganic reductant, also carried out the reaction[1]. Moreover, the CO₂ fixation products, α-keto acids, were successfully converted to the corresponding α-amino acids on treatment with pyridoxamine. It is worth noting that all of these reactions can be carried out in one step as shown in equation (2).

$$RCH_2COH \longrightarrow RCH_2CSC_8H_{17} \xrightarrow[\substack{Schrauzer's\ complex \\ Na_2S_2O_4,\ NaHCO_3 \\ in\ THF\text{-}MeOH\text{-}H_2O}]{CO_2} \left(RCH_2CCO_2H\right)$$

$$\xrightarrow[\substack{pyridoxamine \\ zinc\ acetate}]{} RCH_2CHCO_2H$$
$$\overset{|}{NH_2}$$

(2)
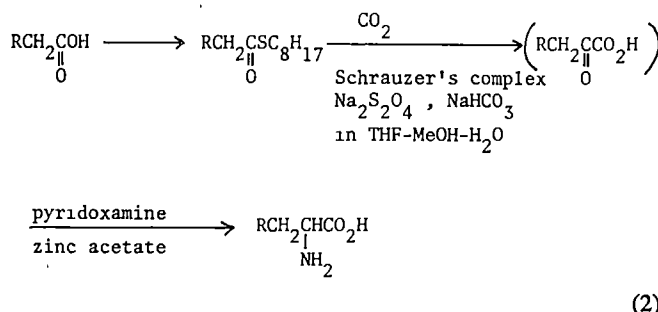
Gaseous CO₂ was bubbled into a solution consisting of tetrahydrofuran (30 ml), methanol (15 ml), water (7.5 ml), synthetic iron–sulphur complex (500 mg, 9.2×10^{-4} mol), sodium hydrosulphite (10 g. 6×10^{-2} mol) and sodium bicarbonate (500 mg, 6×10^{-3} mol). The solution turned dark green, presumably as a result of the formation of the reduced iron–sulphur complex soluble in the solvent. Now, *n*-octyl thiolphenylacetate $C_6H_5CH_2COSC_8H_{17}$ (264 mg, (10^{-3} mol), pyridoxamine dihydrochloride (48.3 mg, 2×10^{-4} mol), potassium hydroxide (22.4 mg, 4×10^{-4} mol) and zinc acetate (55 mg, 2.5×10^{-4} mol) were added to the solution. CO₂ bubbling was continued under efficient reflux and with stirring at room temperature. After 9 h bubbling, the reaction mixture was extracted three times with ether. The aqueous solution was subjected to anion exchange resin chromatography using quarternary ammonium resin.

The products were absorbed on the resin, and eluted with aqueous HCl. The product appeared in the first 1 N-HCl elute, and behaved as authentic phenylalanine, giving a thin-layer chromatography (TLC) spot (silica gel) of the same R_f value with phenylalanine and the same red-purple colour as phenylalanine when treated with ninhydrin. On treatment with CH₂N₂, products gave the same TLC R_f value (silica gel) and red-purple colour as the methyl ester of phenylalanine, and also the same retention time on gas chromatography. Phenylalanine formation in the present reaction was confirmed through coinjection with authentic methyl ester of phenylalanine, infrared and mass spectroscopy.

Thus, the synthesis of phenylalanine by CO₂ fixation with completely artificial (non-enzymatic) catalysts was achieved without addition of any subcellular material. The formation of phenylpyruvic acid was also fully confirmed as phenyl-hydrazone (TLC, infrared spectroscopy and nuclear magnetic resonance) and its methyl ester (TLC). This strongly indicates that phenylalanine was formed from phenylacetic acid (or more favourably from its thiol ester) by way of phenylpyruvic acid (as a reductive CO₂ fixation product) by amino transfer from pyridoxamine as shown in equation (2).

Alanine and leucine were similarly synthesised from corresponding thiol esters of carboxylic acids; the structures of amino acids thus formed were ascertained by TLC and vapour phase chromatography (VPC) after converting them into the appropriate derivatives.

Control experiments were carried out where each of the important components in turn was omitted from the complete system which consists of thiol ester of phenylacetic acid, Schrauzer's complex, sodium hydrosulphite, CO₂ gas,

and amino transfer reagents—pyridoxamine dihydrochloride, potassium hydroxide and zinc acetate. In these control experiments, no trace of phenylalanine was detected. These results show that all of the important components are necessary for the synthesis of an α-amino acid.

The possibility of contamination especially with α-amino acid and/or α-keto acid in all reagents used (whether prepared by us or not) was excluded.

The iron–sulphur complex used in the present non-enzymatic system involves the so-called "Schrauzer's complex," first synthesised by G. N. Schrauzer and its structure in the oxidised state is shown in equation (3).
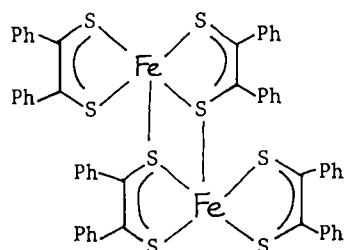
Schrauzer's complex

(3)

The complex showed the same melting point and characteristic infrared spectrum as reported[2]. The insoluble complex was easily reduced with sodium hydrosulphite and the reduced form was soluble in the present solvent. The reduced form has λ_{max} at 314 and 443 nm.

The electronic spectra of Yang's complex[3]. Holm's complex[4] and cysteine-containing peptides[5] were shown to be very similar to those of ferredoxin, and characteristics of reduced iron–sulphur proteins such as their ENDOR and ESR spectra[8], magnetic susceptibility[6] and redox potential[4] were approximately reproduced by artificial iron–sulphur complexes. Our success in a fully artificial CO_2 fixation using Schrauzer's complex may be caused by the similarity of electronic structure between artificial iron–sulphur compound and iron–sulphur protein.

The present non-enzymatic reaction is novel and will aid understanding of the chemical nature of the biological CO_2 fixation process. It is true that this non-enzymatic reaction gives only a low yield, but it is still close to the yield of *in vitro* enzymatic CO_2 fixation. The yield of pyruvic acid was 0.66% based on acetyl phosphate used. The yield in the present reaction was 0.3% based on pyridoxamine used. Some attempts to improve the yield of non-enzymatic CO_2 fixation are now in progress.

T. NAKAJIMA
Y. YABUSHITA
I. TABUSHI

Department of Pharmaceutical Science,
Kyushu University, Katakasu,
Fukuoka 812, Japan

1 Evans, M. C., and Buchanan, B. B., *Proc. natn. Acad. Sci. U S.A.*, 53, 1420 (1965)
2 Schrauzer, G N., Mayweg, V. P , Finck, H. W., and Heinrich, W., *J Am. chem Soc*, 88, 4604 (1966).
3 Yang, C. S., and Huennekens, F. M., *Biochemistry*, 9, 2127 (1970).
4 Averill, B. A., Herskovitz, T., Holm, R H., and Ibers, J. A., *J. Am. chem. Soc*, 95, 3523 (1973); 96, 4159, 6042 (1974)
5 Sugiura, Y, and Tanaka, H , *Biochem biophys Res Commun*, 46, 335 (1972).
6 Rubinson, K. A., and Palmer, G., *J. Am. chem. Soc.*, 94, 8375 (1972).

Seasonal rhythm in plasma testosterone and luteinising hormone of the male laboratory rat

SEASONAL reproductive rhythms of animals living in natural conditions[1–4] as well as domestic animals[5,6] have been the subject of extensive research. Seasonal rhythms in experimental laboratory animals living in a constant laboratory environment, however, have been considered improbable. The first report on seasonal rhythms in laboratory animals was made in 1958 by Gunn and Gould[7], who reported a seasonal cycle in the uptake of ^{65}Zn by the dorsolateral prostate of the male rat, with elevations in February–March and June–July. Comments on the possibility of seasonality of androgenic hormones[8,9] only emphasise that no study has been reported on the male laboratory rat to determine whether there are seasonal variations in plasma testosterone and luteinising hormone (LH) throughout the year. We began such a study in January 1973 and report data collected up to June 1974, which suggest a circannual rhythm in sex hormones in male laboratory rats living in what is apparently a constant environment.

At the time the study began, eight rats of Sprague–Dawley descent were placed in a windowless vivarium with lights on from 0600 to 1800 (eastern standard time). Temperature was maintained at 21 ± 1 °C; Purina rat chow and water were provided *ad libitum*. The animals lived in the experimental environment for 4 weeks before the first blood was collected on February 15, 1973, when the rats were 85 d old. Approximately 1 ml of blood was collected monthly by cardiac puncture under ether anaesthesia. Blood was collected between 1000 and 1200. Other rats of similar birthdate and treatment were added to the study during the first nine months to replace animals lost while collecting blood. On November 1, 1973, animals more than four months old were added to replenish the experimental group, which was badly depleted due to loss of ageing animals. Plasma testosterone and LH levels were also measured in a control group (age control) of six 90-d-old and six 100-d-old males during the spring of 1974, to evaluate the effect of age on seasonal changes in hormone levels. Testosterone concentration was measured by radioimmunoassay using a method we developed[10], LH concentration by an ovine-ovine rat radioimmunoassay[11].

In the 18-month study of rats more than 80 d old, plasma testosterone concentration showed a significant elevation in February–March, 1973 (designated the spring surge) (Fig. 1). There was no spring surge, however, in 1974 in animals more than 180 d old, although plasma testosterone levels were elevated in the younger rats (age control) (Fig. 1). LH levels also rose significantly in the spring of 1973, but did not rise in older male rats in the spring of 1974 (Fig. 1), although the concentration of LH was elevated in the age control group.

The rise in plasma testosterone in 85-d-old rats during February 1973 and 130-d-old rats during March 1973 in our 18-month study contrasted with a report by Grota[12], who described a testosterone surge in 60-d-old rats after which the hormone decreased sharply as the animals aged. Since Grota did not report the birthdate of his experimental animals, we repeated his study in males born in the spring (April 22, 1974) and autumn (October 15, 1973).

In rats which matured in the summer, plasma testosterone levels increased at 61 d of age (June 22) and then decreased (Fig. 2), as Grota reported. In animals born in the autumn, however, plasma testosterone levels increased at 50 d of age, decreased at 60 d, and then increased sharply at 90–100 d (Fig. 2), during January 1974. Apparently the seasonal surge which we observed in our original experiment was based on the fact that in rats born in the autumn, the 60-d plasma testosterone surge was delayed until spring.

The thesis that the 60-d plasma testosterone surge is subject to seasonal regulation was tested further by examining plasma testosterone levels in successive groups of rats, reaching 60–65 d of age during each month of the year except April. A 60-d surge was noted in all months but June and December (Fig. 3), if we define a surge as >3 ng ml^{-1}. Although the relatively low levels of plasma
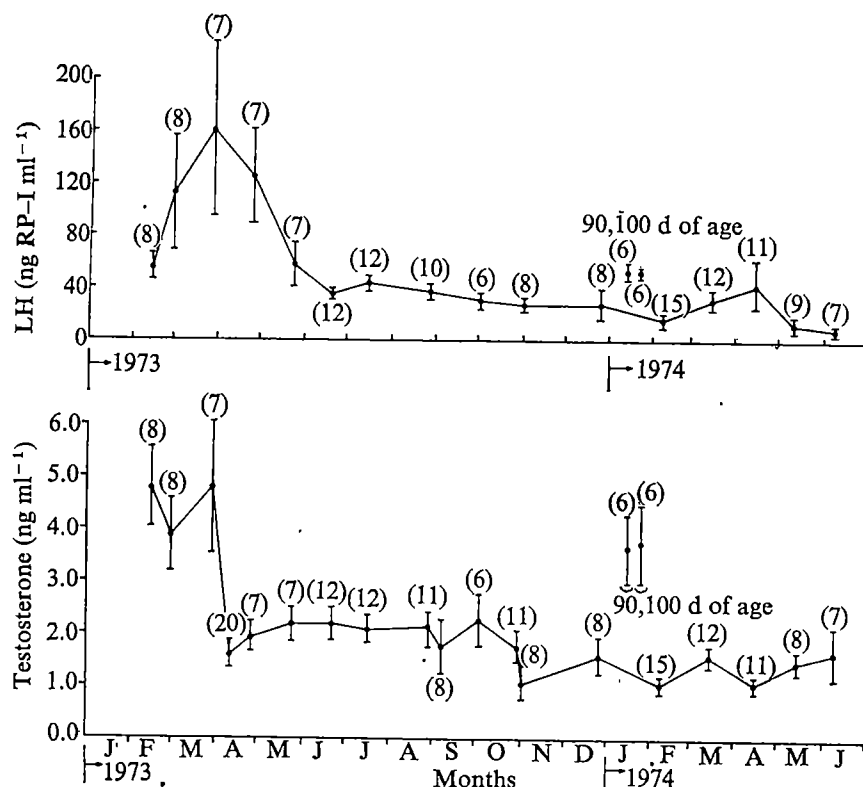
Fig. 1 Plasma testosterone (lower) and LH (upper) concentrations of mature male rats (>85 d of age) over 18 months. Each point in the figure represents ng ml^{-1}, mean±s.e.; the number of animals tested is given in parentheses above each point. Rats were purchased from Russell Miller Farms, at 50 d of age, and reared in vivaria under constant conditions, as described in the text. Blood was collected by cardiac puncture between 1000h and 1200h under light ether anaesthesia. Testosterone and LH were measured by radioimmunoassay[10,11]. Statistical analysis (ANOVA and Student–Neuman–Keuls test) showed that plasma testosterone was significantly increased ($P<0.05$) during February and March 1973 over all other sampling times. Plasma LH was significantly increased ($P<0.05$) during March 1973 over all other sampling times. In the age control group of 90-d-old and 100-d-old animals tested during January 1974, hormone levels were significantly increased ($P<0.05$) compared with other sampling periods of older animals during 1974.

testosterone of 60-d-old rats in June did not represent a delay but rather a diminution of the 60-d surge (Fig. 2), the low levels of plasma testosterone of 60-d-old male rats in December did represent a delay in the surge, which reached a maximum in January 1974, when the rats were 90–100 d old (Fig. 2).

Rats older than 5 months did not respond in the spring, but instead their previous 60-d surge showed increasingly higher levels of plasma testosterone depending on their date of birth (compare Fig. 3, July to November). We investigated whether older rats could respond to gonadotrophins by injecting 0.1 IU human chorionic gonadotropin (HCG) per 100 g body weight into 8-month-old male rats and comparing their plasma testosterone levels over 2-h with those of HCG-injected 3-month-old rats (Table 1). Although we found testosterone concentrations consistently lower in aged rats, both groups responded significantly to HCG stimulation.

Apparently the hormonal seasonal surge was not based on testicular competence but reflected a complex regulation which is not solely dependent on environmental cues. The delay in the 60-d surge of animals born in the autumn is reminiscent of delayed sexual maturation of the field rodent during the winter[13]; in fact we recently observed a stunting of body weight during the growth of autumn-born animals

during the winter. It could be argued that the vivarium's "constant environment" contained hidden cues (in part-
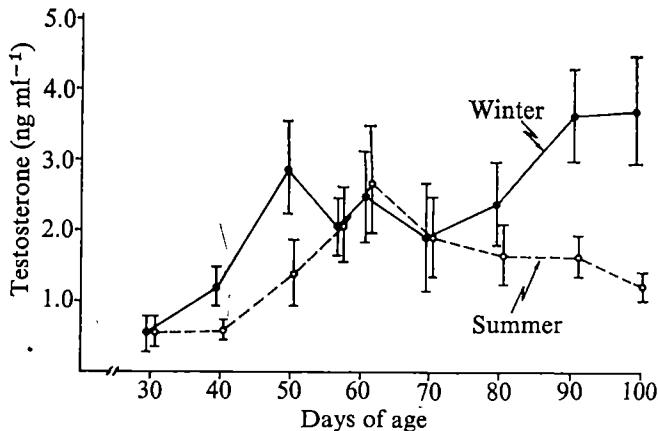
Fig. 2 Plasma testosterone concentrations of maturing male rats in summer (O) (born April 22), and in winter (●) (born October 15). Each point represents the ng ml^{-1}, mean ±s.e. of six animals. Animals were purchased from Russell Miller Farms at 21 d of age and maintained in our vivaria in constant conditions as described in the text.

Table 1 Effect of HCG on plasma testosterone levels of young and old male rats

Group	Treatment	Control sample	Time (min) after injection 15	30	60	120
Young	Saline	1.17±0.34	1.33±0.31	1.47±0.42	1.66±0.42	2.11±0.37
Young	HCG	1.01±0.07	1.85±0.29	3.42±0.38	5.48±0.75	6.96±0.91
Old	Saline	0.85±0.17	0.95±0.13	0.89±0.13	0.72±0.15	0.86±0.22
Old	HCG	0.65±0.15	0.92±0.08	2.04±0.42	3.78±0.44	5.79±0.71

Figures are ng ml^{-1}, mean ±s.e. three-month and 8-month-old male rats were cannulated with PE-50 tubing in the right external jugular vein 24 h before initiation of the experiment. 0.25 ml of blood was collected 3-10 min before treatment (control sample). 0.1 IU of HCG per 100 g body weight or an equivalent volume of physiological saline was then injected into the cannula which was rinsed with 0.2 ml of saline. Blood (0.25 ml) was collected 15, 30, 60 and 120 min after injection. Analysis of variance showed a significant increase ($P<0.005$) in plasma testosterone levels after the injection of HCG. There was no difference in responses between young and old rats. The experiment was run between 1230 and 1600 during August 1974. Animals were purchased from Russell Miller Farms 30 d before the experiment began and maintained in our vivaria in constant conditions as described in the text.
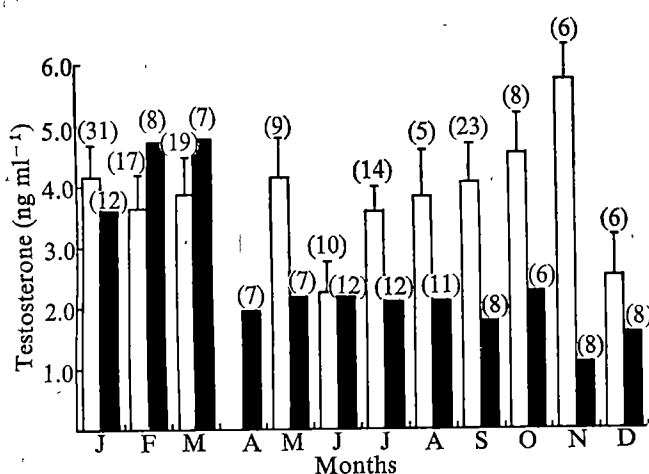
Fig. 3 Comparison of plasma testosterone levels of mature (solid bar) and 60-d-old to 65-d-old (open bar) male rats in different months of the year. The number of animals tested is within the parentheses above each bar. Mean values are graphed; s.e. is included for young animals only. Data of mature rats are from Fig. 1. Rats were bred in our vivaria from the same stock as described in Fig. 1, and reared in constant conditions as described in the text. Analysis of variance showed a significant difference between ages ($P < 0.01$), among months ($P < 0.025$) and a significant age by month interaction ($P < 0.01$).

icular, seasonal fluctuations of plant oestrogens in animal food). It is difficult to sustain this thesis. First, we obtained similar results over several years, using both home-bred and commercially-bred animals which grew up in different environments with different food; second, animals of different birth dates and ages but living in the same evironment responded differently to seasonal influences; third, close examination of two recent reports[14,15] indicates that female rats are also seasonally regulated; and fourth, our animal food purchases and storage were unscheduled.

This report provides the first experimental backing to a growing realisation among scientists that experimental results may differ, depending on the season of the year, particularly when hormonal events with circadian rhythmicity are being examined[16]. While the concept that seasonality may be based on internal factors and not on changing environmental conditions is difficult to accept, it now has experimental as well as ideological support[17,18].

Our research was supported in part by grants from the NIH. We thank the following for donation of materials used in radioimmunoassays: Dr B. V. Caldwell, Dr G. D. Niswender, Dr L. E. Reichert, Jr, and the NIH.

EDWARD J. MOCK
FREJA KAMEL
WILLIAM W. WRIGHT
ARTHUR I. FRANKEL

Department of Biological Sciences,
State University of New York at Binghamton,
Binghamton, New York 13901

1 Amoroso, E. C., and Marshall, F. H. A., in *Marshall's Physiology of Reproduction,* third ed , 1, part 2 (edit. by Parkes, A. S.), 707 (Longmans Green, London, 1960).
2 Sadleir, R. M. F. S., *The Ecology of Reproduction in Wild and Domestic Mammals* (Methuen, London, 1969).
3 Asdell, S. A , *Patterns of Mammalian Reproduction,* second ed (Cornell University Press, Ithaca, 1964).
4 Bullough, W. S., *Vertebrate Reproductive Cycles,* second ed (Methuen, London, 1961).
5 Clegg, M. T., and Ganong, W. F , in *Reproduction in Domestic Animals,* second ed. (edit. by Cole, H. H., and Cupps, P. T.), 473 (Academic, New York, 1969).
6 Ortavant, R., Mauleon, P., and Thibault, C., *Ann. N.Y. Acad. Sci.,* 117, 157-193 (1964)
7 Gunn, S. A., and Gould, T. C , *J. Endocr.,* 17, 344-348 (1958).
8 Ellis, L C , in *The Testis,* 3 (edit. by Johnson, A. D., Gomes, W. R., and Vandemark, N. L.), 346 (Academic, New York, 1970).
9 Kinson, G. A., and Liu, C., *J. Endocr.,* 56, 337-338 (1973).
10 Frankel, A. I., Mock, E J., Wright, W. W., and Kamel, F., *Steroids* 25, 73-98 (1975).
11 Niswender, G. D., Midgley, A. R. Jr., Monroe, S. E., and Reichert, L. E. Jr., *Proc. soc. Exp. Biol. Med.,* 128, 807-811 (1968).
12 Grota, L. J., *Neuroendocrinology,* 8, 136-143 (1971).
13 Christian, J. J., *Proc. natn. Acad. Sci. U.S.A ,* 47, 428-449 (1961); *Biol. Reprod.,* 4, 248-294 (1971).
14 Ramaley, J. A., and Bunn, E. L., *Endocrinology,* 91, 611-613 (1972).
15 Purvis, K., Cooper, K. J., and Haynes, N. B., *J. Reprod. Fert.,* 27, 167-176 (1971).
16 von Mayersbach, H., in *The Cellular Aspects of Biorhythms* (edit. by von Mayersbach, H.), 87 (Springer, New York, 1967).
17 Aschoff, J., *Cold Spring Harb. Symp. quant. Biol.* 25, 11-26 (1960).
18 Schwartz, N. B., in *Handbook of Physiology,* sect. 7, 2, part 1 (edit. by Greep, R. O.), 125 (American Physiological Society, Washington DC, 1973).

An approach to amino acid sequence analysis of transplantation antigens

THE major histocompatibility complex of the mouse, known as the H-2 locus, represents the prime barrier against successful tissue transplants[1] and controls the expression of many cell surface alloantigens which are serologically detectable[2,3]. It may be subdivided by recombinational analysis into four major regions known as K, D, Ir and Ss (ref. 4). The Ss region controls the quantitative expression of a serum protein[5]. The Ir region controls immune responsiveness of mice to a variety of synthetic and natural antigens[6,7], and the expression of multiple cell surface proteins, some of which may be distinguished by molecular weight[8,9]. The K and D regions both control the expression of distinct cell surface glycoproteins (molecular weight 47,000), which are non-covalently associated with a small molecular weight (11,500) component[10-11]. Because the native molecules are hydrophobic proteins present on the cell surfaces in small quantities, it is necessary to use unusual procedures for their isolation and chemical characterisation. We have, therefore, used indirect immunoprecipitation and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) for the isolation and subsequent amino acid sequence analysis of both molecular weight components.

Cell surface proteins of B10.D2 spleen lymphocytes were

Fig. 1 Preparations of the small molecular weight component which had been intrinsically radiolabelled with ^{3}H-tyrosine ($\triangle$) or ^{3}H-lysine ($\bullet$) were combined and analysed on an automated sequenator. The PTH–amino acid derivative from each cycle was combined with an appropriate mixture of unlabelled PTH–amino acids and separated by one dimensional thin layer chromatography on silica gel plates (Eastman–Kodak) using benzene–acetic acid (9:1) as the chromatographic solvent. PTH–amino acid spots were visualised by short-wave ultraviolet light, excised from the plate, and counted in a liquid scintillation counter. The amount of radioactivity associated with each of the PTH–amino acids is plotted against the step number.
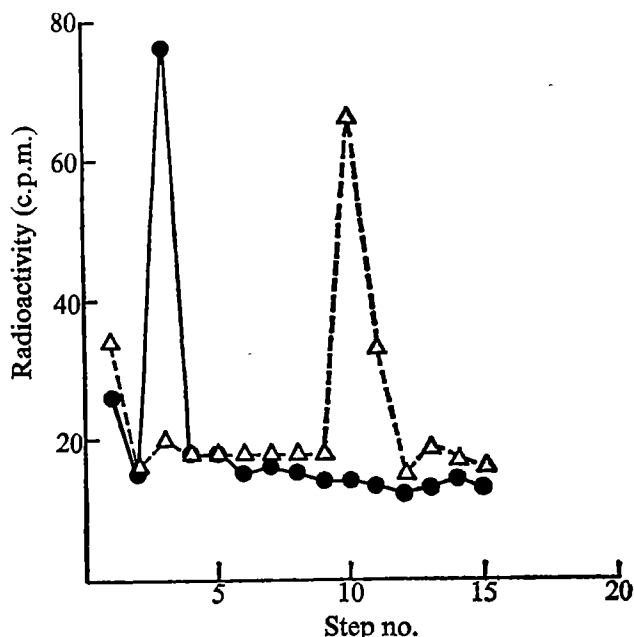
Table 1 Amino acid sequences of B_2-microglobulin

	1	2	3	4	5	6	7	8	9	10
Human	Ile	Gln	Arg	Thr	Pro	Lys	Ile	Gln	Val	Tyr
Dog	Val	Gln	His	Pro	Pro	Lys	Ile ·	Gln	Val	Tyr
Rabbit	Val	Gln	Arg	Ala	Pro	Asn	Val	Gln	Val	Tyr
H-2 associated polypeptide	—	—	Lys	—	—	—	—	—	—	Tyr

radiolabelled by incorporation of single tritiated amino acids. The H-2.4 alloantigen (product of the H-2D region) and its associated polypeptide were isolated by indirect immunoprecipitation using specific H-2 alloantiserum, and purified on SDS PAGE gels as described previously[11]. Each molecule was eluted from the gel by overnight incubation of appropriate gel slices in 0.01% SDS. When re-electrophoresed on SDS gels, the eluted material migrated as a single molecular weight peak. Before sequence analysis, each sample was dialysed twice against 0 001% SDS. Sequential protein degradation was performed on a Beckman Model 890 sequencer using the procedure of Hermodson[12]. Radioactive phenylthiohydantoin (PTH) derivatives were analysed by thin-layer chromatography as described in Fig. 1.

Sequence analysis of the small molecular weight component enabled the assignment of lysine and tyrosine as the amino acid residues to positions 3 and 10 respectively (Fig. 1). A comparison of our sequence assignment with the amino acid sequence of human[14], rabbit[15] and dog[16] B_2-microglobulin isolated from urine (Table 1) supports our previous suggestion[11] that the H-2 associated polypeptide is analogous to the B_2-microglobulin-like molecule found associated with detergent solubilised HL-A alloantigens[13]. Recent preliminary data from our laboratory have enabled us to identify valine as the amino acid residue at position 9 in the small molecular weight component. In addition, rat B_2-microglobulin has been shown by other investigators to have a lysine residue at position 3 (ref. 17). Both findings further support this suggestion.

Amino acid sequence analysis of the H-2.4 alloantigen by this procedure enabled the assignment of leucine and tyrosine residues to position 5 and 7 respectively (Fig. 2). The observed low experimental yield (approximately 40% of theoretical) is probably caused by protein 'washout' from the sequenator cup, and loss of exchangeable tritium.
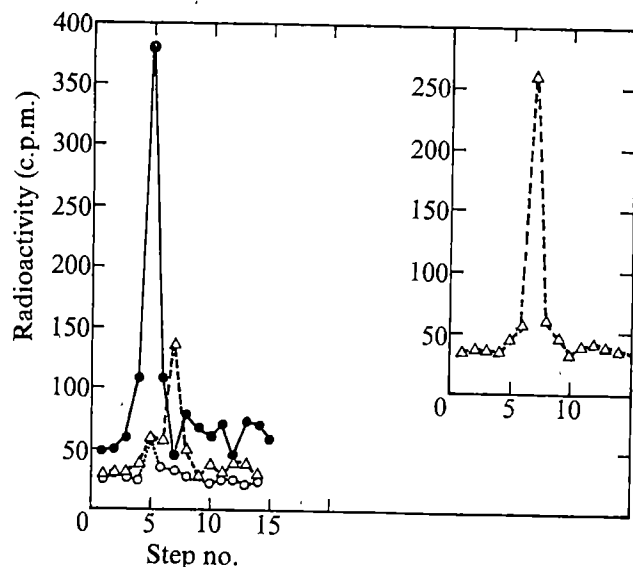
Nevertheless our data suggest that the sequence obtained is representative of a major component of the purified material, and that the H-2.4 alloantigen does not have a blocked N terminus.

These sequence data were obtained from protein which had been isolated from two mouse spleens or approximately 0.15 nmol protein, 1/1,000 the amount normally required for automated sequence analysis. Our results suggest that the H-2 alloantigens as well as other membrane proteins may be amenable to this type of sequence analysis.

Determination of the chemical structure of H-2 and other histocompatibility alloantigens as well as the elucidation of the chemical basis of their antigenicity has implications for tissue and organ transplantation. For example, use of such knowledge may make it possible to render prospective organ recipients specifically tolerant to the unique histocompatibility alloantigens of the prospective donor. In addition, further chemical analysis may clarify the function of the histocompatibility alloantigens and contribute to our basic understanding of cell membrane proteins.

This work was supported by a grant from the NIH.

J. SILVER
L. HOOD

Division of Biology,
California Institute of Technology,
Pasadena, California 91125

1 Counce, S., Smith, P, Barth, R., and Snell, G. D., Ann Surg., 144, 198–204 (1956).
2 Amos, D B., Gorer, P. A., and Mikelska, Z. B., Proc. R. Soc, B144, 369–380 (1955).
3 David, C. S., Shreffler, D C., and Frelinger, J. A, Proc. natn. Acad Sci. U.S.A, 70, 2509–2514 (1973).
4 Klein, J., and Shreffler, D. C., Transplant. Rev., 6, 3–29 (1971)
5 Shreffler, D. C., and Passmore, H. C., in Proc. Symp. Immunogenet. H-2 Sys., 58–68 (S. Karger AG, Basel).
6 McDevitt, H. O, et al., J. exp Med, 135, 1259-1278 (1972).
7 Lieberman, R., and Humphrey, W. J., J. exp. Med, 136, 1222–1230 (1972).
8 Cullen, S E., David, C S., Shreffler, D. C., and Nathenson, S. G., Proc. natn. Acad Sci. U.S.A, 71, 648–652 (1974).
9 Silver, J., Sibley, C., Morand, P., and Hood, L., Transplant. Proc. (in the press).
10 Schwartz, B D, Kato, K., Cullen, S. E., and Nathenson, S. G., Biochemistry, 12, 2157–2164 (1973).
11 Silver, J., and Hood, L, Nature, 249, 764–765 (1974)
12 Hermodson, M. A, Dricsson, L. H., Titani, K., Neurath, H., and Walsh, K. A, Biochemistry, 11, 4493–4501 (1972).
13 Grey, H M., et al, J exp. Med., 138, 1608–1612 (1973)
14 Cunningham, B. A, Wang, J. L., Berggard, I., and Peterson, P. A., Biochemistry, 12, 4811–4822 (1973)
15 Cunningham, B A., and Berggard, I., Science, 187, 1079–1080 (1975).
16 Smithies, O, and Poulik, M. D, Proc. natn. Acad Sci U.S A., 69, 2914–2917 (1972).
17 Poulik, M. D., Shinnick, E. S, and Smithies, O., Fedn. Proc., 34, 945 (1975)

Fig. 2 Amino acid sequence analysis of the H-2.4 alloantigen radiolabelled with ^{3}H-leucine (●), ^{3}H-tyrosine (△), and ^{3}H-threonine (○) was carried out as described in Fig. 1. The results of a second sequenator run of ^{3}H-tyrosine-labelled H-2.4 alloantigen is shown.

Stable large variant of 5S RNA in Clostridium thermosaccharolyticum

THE precursor forms of prokaryote 16S and 23S ribosomal RNA (rRNA) are larger than their mature counterparts by some 10% (refs 1–5). An analogous large precursor form of the bacterial 5S RNA exists (in at least some cases)[1,3,6,7]. The reason for the existence of these rRNA precursor forms is not known. They could merely reflect an aspect of the transcription process, or they could have evolved specifically to facilitate ribosome assembly. A more intriguing possibility is that they represent vestiges of ancestral rRNAs, retained because they are essential to ribosome assembly—which process must reasonably recapitulate, to some extent, ribosomal evolution. This last alternative gains some support from our

discovery of a stable form of the 5S RNA some 30–40% larger than normal, in *Clostridium thermosaccharolyticum*.

Polyacrylamide gel profiles of ^{32}P-labelled RNA from *C. thermosaccharolyticum* exhibit an extra band that corresponds to an RNA species of about 160 nucleotides. The new species plus the normal 5S RNA together exist in approximate unit mole ratio to the 16S and 23S rRNAs. The new species is indeed RNA and stable because a high specific activity pulse of ^{3}H-uridine (>90% incorporated in less than one cell generation) is incorporated into it; and the ratio of ^{3}H in the new RNA species to that in 5S RNA remains constant (about unity) for at least four generations (the length of the experiment).

Figure 1 is a polyacrylamide gel profile of RNA extracted from the 50S subunit. Both normal 5S RNA and the new RNA species are found on this subunit. Although all this evidence may suggest the new RNA species to be of the 5S RNA genre, definitive proof depends on some more detailed characterisation, such as oligonucleotide fingerprinting.

Figure 2 shows tracings of the two-dimensional electro-phoretograms of T1 ribonuclease digests of both the normal 5S RNA (form I) and the new species (form II) from *C. thermo-saccharolyticum*. Table 1 catalogues the oligomers found in both T1 and pancreatic ribonuclease digest of the two forms.

Form II contains (almost) every oligonucleotide found in form I. We conclude, therefore, that form II is a '5S' RNA. Form II also has an additional forty or so nucleotides not found in form I, giving the former an approximate size of 160 nucleotides. Both forms contain the same 5' hexanucleotide, pUUUCCG. The 3' terminal oligonucleotide of the shorter form I, however, is absent in form II. The simplest interpretation of this is that the excess material unique to form II occurs as an extension of the 3' terminus of the normal molecule. The presence of several potential stable base-pairing sequences—GGGGU to [C$_{4-5}$U]G and ACUUUUUUG to GAAAAGU—predicts that the excess material in form II will possess considerable secondary structure.
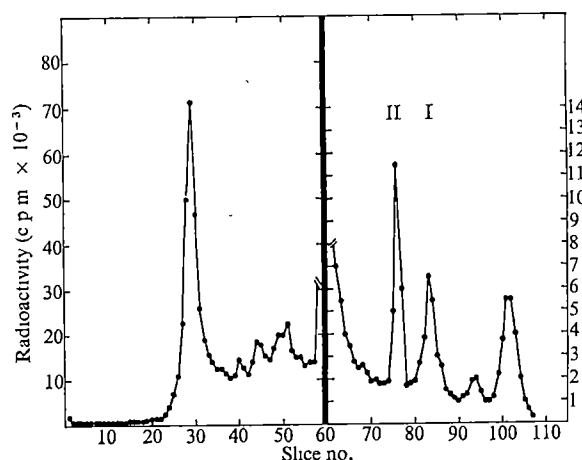
Fig. 1 Polyacrylamide gel electrophoretic profile of RNA from 50S subunit of *C. thermosaccharolyticum*, grown anaerobically in 5 ml dephosphorylated yeast extract–peptone medium to which Na$_2$S and cysteine were added[8]. Approximately 100 µCi of [32]PO$_4$ were added to the culture early in log phase and the culture collected three to four generations later, by low speed centrifugation (12,000g for about 10 min). Cells were ruptured by passage through a French press, and the lysate, cleared of all debris—again by low speed centrifugation—was layered on a 5–20% sucrose gradient in a 10 mM Tris buffer, pH 7.4, containing 0.1 mM MgCl$_2$. After centrifugation (90,000g for 9 h in a Spinco SW 25.1 rotor), the resulting 50S peak was collected, dialysed to remove sucrose, and the RNA extracted by the phenol method[9], the purified RNA being subsequently analysed by polyacrylamide gel electrophoresis. A split gel method was used[10], the initial portion comprising 3.2% acrylamide (a), the final portion, 10% acrylamide (b). The large peak in the 3.2% gel section has the mobility one would expect for 23S rRNA; the peaks labelled I and II in the 10% section of the gel have the mobilities expected for normal 5S RNA and the new RNA species, respectively. The remaining material in the 10% section of the gel is presumably tRNA and/or 23S rRNA breakdown products.

Table 1 Sequences and molar occurrence of oligomers (dimer and larger) produced by T1 or pancreatic ribonuclease digestions of forms I and II of *C. thermosaccharolyticum* 5S RNA

	T1 ribonuclease			Pancreatic ribonuclease		
Oligomer	I	II	Oligomer		I	II
CG	2 1/2	2 1/2		AC	4	4
AG	4	3 1/2		AU	1 1/2	2 1/2
UG	2 1/2	3 1/2		GC	3	4 1/2
				GU	1 1/2	2
CCG	2	2		AGC	2	2 1/2
AAG	2 1/2	1		GAU	2	3
UCG	1	1 1/2		AGU	0	1
CUG	1	1		GGC	1	1
UAG	1	1		GGU	2	4
AUG	1	1		AAU	1	1
CAAG	0	1		GAAC	1	1
AUAG	0	1		AAAU	0	1
				GAGU	1/2	0
AAAAG	0	1		AGGU	1	1
UACUG	1	1				
AACACG	1	1		AAGAC	0	1
CAAUAG	1	1		GAAGC	1	1
				GGGGU	0	1
[C$_{4-5}$U]G	0	1		AAAAAC	1	1
CCC(CU)CAG	1	1		GGAAGU	1	0
AUUACUCG	1	1		GG[G, AG]U	1	1
ACUUUUUUG	0	1		GAAAAGU	0	1/2
(UCCC)AAAUCG	0	1				
				GGGAGAGU	1	1
UUAAAAACCCG	1	1				
U(UC)CCAUUCCG	1	1		pU	1	1
pUUUCCG	1	1		(AG)C$_{OH}$*	0	1
AUAAU$_{OH}$	1/2	0				

Oligomer spots shown in Fig. 2 and comparable spots from pancreatic nuclease fingerprints were sequenced as described previously[12-14]. Where order is uncertain in an oligomer, the nucleotides in question are in parentheses. Quantification by scintillation counting of excised spots in a non-aqueous scintillant (BBOT) are given to the nearest 0.5 mol ratio.
*Identification not certain.

A possible explanation for form II is that it is related, at least in an evolutionary sense, to 5S precursor forms[6,7]. Sogin and Pace have shown that the conversion from precursor to

mature 5S RNA (in Bacilli) involves cleavage by the same enzyme at points defining both the 5' and 3' termini of the mature 5S RNA (ref. 7). An identical large purine stretch is found about 20 nucleotides proximal to the point of cleavage in each instance, and presumably therefore, serves as a recognition signal for the enzyme, RNase m5 (ref. 7). Were this the general mechanism for 5S RNA maturation (in prokaryotes), it would demand in the present case that form II not contain such a signal defining a normal 3'-terminal cleavage point. Indeed, form II does not possess one large purine stretch, GGAAGU, found in form I, but its position in the molecule is unknown at present.

The existence of a large form of the 5S RNA raises many questions regarding both function and evolution in the ribosome. The trivial explanation that the large form of 5S RNA has neither functional nor evolutionary significance cannot be ruled out, but it seems unlikely. Ratios of the two forms of 5S RNA from cells grown at the extremes of the organisms' growth range (that is, 45 and 65 °C) are not significantly different, ruling out the possibility that one form may be used preferentially at higher growth temperatures. We have not determined whether this ratio varies during sporulation.

As yet, the stable large form of 5S RNA is unique to *C. thermosaccharolyticum*. It is definitely not present in Bacilli[6,7], and we have looked for, but failed to find it in mesophilic Clostridia, even in representatives of the same subgroup classification as *C. thermosaccharolyticum* (K. Luehrsen and C.W., unpublished).

Two explanations remain. It could be a retention of or return to an ancestral type of 5S RNA—the latter being made possible through retention of ancestral characteristics in precursor forms of the 5S molecule. On the other hand, it may represent a novel evolutionary happening, having no resemblance to archaic forms of 5S RNA. We do not favour the latter on the grounds that the change in passing from the normal to enlarged form of 5S RNA is a drastic one (and would probably require further evolutionary changes in the ribosome), and it would seem *a priori* unlikely to occur in a fully evolved ribosome (that is, in one species of one genus).

This work was supported by a grant from the US Public Health Service to C.W. We thank Dr G. Fox for discussions.

L. A. SUTTON
C. R. WOESE

*Departments of Microbiology
and Genetics and Development,
University of Illinois,
Urbana, Illinois 61801*

1 Hecht, N. B., and Woese, C. R., *J. Bact.*, 95, 986–990 (1968).
2 Adesnik, M., and Levinthal, C., *J. molec. Biol.*, 46, 281–286 (1969).
3 Doolittle, W. F., *J. Bact.*, 111, 316–320 (1972).
4 Sogin, M., Pace, B., Pace, N. R., and Woese, C. R., *Nature*, 232, 48–49 (1971).
5 Sogin, M. L., Woese, C. R., Pace, B., and Pace, N. R., *J. molec. Evol.*, 2, 167–174 (1973).
6 Pace, N. R., Pato, M. L., McKibbin, J., and Radcliffe, C. W., *J. molec. Biol.*, 75, 619–625 (1973).
7 Sogin, M. L., and Pace, N. R., *Nature*, 252, 598–602 (1974).
8 Sogin, M. L., Pechman, K. J., Zablen, L., Lewis, B. J., and Woese, C. R., *J. Bact.*, 112, 13–16 (1972).
9 Gierer, A., and Schramm, G., *Nature*, 177, 702–708 (1956).
10 Sogin, S., thesis, Univ. Illinois (1971).
11 Cory, S., Adams, J. M., Spahr, P-F., and Rensing, U., *J. molec Biol.*, 63, 41–56 (1972).
12 Sanger, F., Brownlee, G. G., and Barrell, B. G., *J. molec. Biol.*, 13, 373–398 (1965).
13 Brownlee, G. G., *Determination of Sequences in RNA* (North-Holland, Amsterdam and London, 1972).
14 Uchida, T., et al, *J. molec. Evol*, 3, 63–77 (1973).

Fig. 2 Tracings of two-dimensional electrophoretograms of T1 ribonuclease digests of 5S RNA (form I) and the new RNA species (form II). *C. thermosaccharolyticum* was grown, labelled, and cells ruptured as described in Fig. 1, except that 5-10 mCi $^{32}PO_4$ were added to the culture. Cell lysate was extracted immediately by the phenol method[9], and the resulting RNAs isolated by electrophoresis on 10% polyacrylamide gels. Appropriate RNA bands were located by radioautography of the intact gel, the bands cut out, and the RNA extracted by phenol[11]. Specific activities of the RNA varied from preparation to preparation, but were within the range 0.5–2 µCi per µg RNA. The two RNAs were purified by passage over BD-cellulose[10], digested with T1 ribonuclease and the resulting products analysed by a modification of two-dimensional electrophoretic method[12-14]. The first electrophoretic dimension was run on cellulose acetate at *p*H 3.5, the second on DEAE-cellulose paper in a 0.1 M pyridine solution brought to *p*H 2.3 with formic acid (ref. 14 and M. Sogin, D. Stahl, L. Bonen and C.W., unpublished). Oligonucleotides unique to Form II are italicised.

Oxygen binding to haemoglobins of the primitive vertebrate *Myxine glutinosa* L.

THE Atlantic hagfish (*Myxine glutinosa* L.) belongs to the family Myxinidiae which forms along with the family Petromyzonidae (the lampreys) the only living class of jaw-

less vertebrates (Agnatha), the Cyclostomata. These Cyclostomata are considered on morphological[1] and physiological[2,4] evidence to be the most primitive form of living vertebrates and are distant descendants of fossil forms of Agnatha which were already present during the Ordovician, that is, more than 400 Myr ago[1,3].

The oxygen-binding characteristics of hagfish haemoglobin offer an opportunity to obtain information on the evolution of the respiratory function of haemoglobin. It seems[4-10] that hagfish haemoglobin, unlike all known haemoglobins of higher vertebrates which carry four haems per molecule, has only one oxygen-binding site. Not unexpectedly they exhibit little, if any, allosteric effects, such as cooperativity of oxygen binding and Bohr effect, and would be therefore called 'primitive' in comparison to more advanced forms of molecular organisation of respiratory proteins represented by tetrameric haemoglobin. If the oxygen-binding characteristics of the haemoglobins of *M. glutinosa* reported here, however, are compared with those of the Pacific[5,6] (*Eptatretus stoutii*) and Japanese[7] (*E. burgeri*) hagfish, it seems that these groups of animals form distinct functional entities and can be called 'primitive' only from a comparative point of view.

Preparation of haemoglobin solutions from the blood of *M. glutinosa* and fractionation of the three major components (Hb$_I$, Hb$_{II}$, Hb$_{III}$) was carried out as described previously[9,10]. We found that the three major types of haemoglobin which are present in the blood of *M. glutinosa* have different oxygen affinities (Fig. 1), but did not show any sign of cooperativity when judged from the exponent n in the Hill equation[13]. Even though Hb$_I$, Hb$_{II}$ and Hb$_{III}$ represent only 52% of the total (the remainder being minor components) they seem to govern the oxygen affinity of the total haemolysate. This can be deduced from the good agreement between the partial pressure of oxygen at which haemoglobin is half saturated with oxygen (P_{50}) measured in the total haemolysate (4.2 mmHg at pH 7.3 and 20 °C) and that calculated from the respective percentage of each component[9] (4.3 mmHg in identical conditions). Hb$_{II}$ and Hb$_{III}$ did not change oxygen affinity in the pH range between 6.85 and 8.20, that is, they did not exhibit the Bohr effect. Note that multiple haemoglobin fractions of *E. burgeri* also showed functional heterogeneiety[7] although the major compounds A, B and C found in *E. stoutii* did not[6]. Thus, there is haemoglobin polymorphism in all three species of hagfish but in only two of them do different molecules

Fig. 2 Plot of log P_{50} against pH of hagfish haemolysate. O, Without CO_2; ●, in presence of CO_2. Note that in these latter experiments pH was changed by varying P_{CO_2} at constant $(23.9 \times 10^{-3}$ M l^{-1}) bicarbonate concentration. Upper part of figure shows the exponent n of the Hill equation[13] in which the lines represent the means obtained in the absence and presence (dotted line) of CO_2. Temperature was 20 °C and haemoglobin concentration 0.1×10^{-3} M l^{-1}. Buffers were 50 mM bis-Tris–100 mM NaCl in the absence of CO_2 and bicarbonate–NaCl buffer with a molarity of 150 mEq l^{-1} in the presence of CO_2. As methaemoglobin was present in some preparations, the samples were reduced with sodium dithionite and the reaction products removed on a mixed-bed ion-exchange column[11] before oxygen-binding experiments[12].

Fig. 1 Plot of log $(y/1-y)$ against log P_{O_2} for the isolated haemoglobins Hb$_I$ (O), Hb$_{II}$ (△), Hb$_{III}$ (□) of hagfish blood (Hill plot[13]). Y represents saturation of haemoglobin with oyxgen. P_{50}: 3.7, 2.3 and 6.3 mmHg for Hb$_I$, Hb$_{II}$ and Hb$_{III}$ respectively. Hill's exponent n averaged 1.03 for the three components. Temperature was 20 °C, pH 7.3 and haemoglobin concentration 0.1×10^{-3} M l^{-1}.

have different oxygen affinities. In particular environmental conditions such differences in haemoglobin function could be of physiological significance. If, for example, ambient temperature is raised, an increased synthesis of Hb$_{II}$ which has a high oxygen affinity (Fig. 1) would counteract the impaired oxygen uptake of the animal brought about by the high heat of oxygenation (-12.7 calorie mol^{-1}) which we determined in the haemolysate of *M. glutinosa*.

Consider the oxygen-binding properties of the whole haemolysate (Fig. 2). In the pH range of physiological importance for hagfish[4,5] (7.5–7.8) there was very little change in oxygen affinity with varying pH (Δlog P_{50}/ΔpH = -0.07) at least in the absence of CO_2, thus confirming the results of Manwell[4]. At pH less than 6.5 we observed a more pronounced Bohr effect which is, however, of no significance for the gas exchange in *M. glutinosa*. Addition of CO_2, on the other hand, significantly decreased oxygen affinity and increased the Bohr effect to -0.17 (Δlog P_{50}/ΔpH) in the range between pH 7.3 and 8.2. This relatively important increase in Bohr effect in the presence of CO_2 is still small in absolute terms and therefore compatible with the notion that fish living in an environment with varying CO_2 pressures (as the burrowing hagfish probably does), have in general a reduced Bohr effect[4,8]. Furthermore, the Hill parameter n increased significantly ($0.02 > P > 0.01$) on addition of CO_2, indicating that cooperativity of oxygen binding increased. This is probably caused by an increased tendency of the various components of *M. glutinosa* haemoglobin to aggregate. Such aggregation, also found in *E. burgeri*[7] and *Petromyzon marinus*[14,15] at low pH and high haemoglobin concentration, may be interpreted as an early attempt of nature to form the cooperative heterotetramers[16], which carry oxygen in the blood of higher vertebrates. In the isolated components Hb$_I$, Hb$_{II}$ and Hb$_{III}$ this specific effect of CO_2 on the oxygen-binding properties was not observed. Hb$_I$ and Hb$_{II}$ have blocked N-terminal amino groups, whereas in Hb$_{III}$ the N terminus is free to form carbamino compounds. This binding of CO_2 to Hb$_{III}$ does not seem to lead to self-aggregation which reflects itself in decreased oxygen affinity.

The question arises therefore how the CO_2 effect can be

explained. If it is not mediated by Hb_{III} there must be one or several minor components whose oxygen affinity is considerably decreased by CO_2. As the haemolysate of *M. glutinosa* contains more than ten of these minor fractions[9,10], none of which has been structurally characterised, it is impossible at present to identify the ones which may indicate the CO_2 effect. Nevertheless, the possibility should be kept in mind that the decrease in oxygen affinity produced by CO_2 could be brought about by interactions between different haemoglobin molecules. Such intermolecular interactions have a significant influence on the oxygen binding properties in the haemolysate of *E. burgeri*, even though the isolated, homogeneous components show practically no tendency to associate[7]. By the same token, it seems possible that the increased surface charge at specific sites in Hb_{III} (or some minor fractions), which is the result of carbamate formation, leads to an aggregate product between different molecules with a consecutive decrease in oxygen affinity of the whole haemolysate. In contrast to CO_2, other effector molecules which have large effects in tetrameric haemoglobin, like 2,3-diphosphoglycerate, inositol hexaphosphate and high concentrations of NaCl had no influence on oxygen affinity and cooperativity of *M. glutinosa* haemoglobin. Even an increase in protein concentration by a factor of six had no effect on P_{50} or the exponent n, in contrast to what is found in *E. burgeri*[7].

If we compare the P_{50} values which have been obtained in different species of hagfish in nearly identical conditions with respect to haemoglobin concentration, pH and temperature, *E. burgeri* has the lowest oxygen affinity $(P_{50} = 10\ mmHg)[7]$ being followed by *M. glutinosa* $(P_{50} = 4.2\ mmHg)$ *E. stoutii* has the highest oxygen affinity $(P_{50} = 1.6\ mmHg)[5,6]$. As long as we know as little as we do on ambient conditions and the respiratory capacity of these animals it would be inappropriate to draw any far reaching conclusion on possible ecological advantages of such differences in oxygen-binding characteristics of hagfish haemoglobin. These particular respiratory proteins seem to be primitive in the sense that they exhibit very limited allosteric effects. Note, however, that hagfish are biologically very successful animals so that their haemoglobins are likely to fulfil their physiological function quite well. Oxygen exchange, for example, takes place at such low internal partial pressures of oxygen in these animals[4] that the absence of significant cooperativity of oxygen binding to haemoglobin does not seem to have any adverse effects on oxygen delivery to the tissues.

C. Bauer
U. Engels

Physiologisches Institut,
Medizinische Hochschule,
3 Hannover, Germany

S. Paleus

Blodverksamheten,
Fack, S–10064 Stockholm, Sweden

1 Heintz, A., in *The Biology of Myxine* (edit. by Brodal, A., and Fänge, R.), 9–21 (Universitetsforlaget, Oslo, 1963).
2 Jensen, D , *Comp. Biochem. Physiol* , 2, 181–201 (1961)
3 Berkner, L. V., and Marshall, L. C *J. atmosph. Sci* , 22, 225–261 (1965).
4 Manwell, C., in *The Biology of Myxine* (edit. by Brodal, A., and Fänge, R.), 372–455 (Universitetsforlaget, Oslo, 1963).
5 Manwell, E., *Biol. Bull.*, 115, 227–238 (1958).
6 Li, S.-L., Tomita, S., and Riggs, A., *Biochim biophys Acta*, 278, 344–354 (1972).
7 Bannai, S., Sugita, Y., and Yoneyama, Y., *J. biol Chem.*, 247, 505–510 (1972).
8 Johansen, K., and Lenfant, C., in *Oxygen Affinity of Haemoglobin and Red Cell Acid Base Status* (edit. by Rørth, M., and Astrup, P.), 750–780 (Munksgaard, Copenhagen, 1972).
9 Paléus, S., Vesterberg, O., and Liljequist, G., *Comp. Biochem. Physiol.*, 39B, 551–557 (1971).
10 Paléus, S , and Liljequist, G., *Comp Biochem. Physiol.*, 42B, 611–617 (1972).
11 Bauer, C , and Pacyna, B., *Analyt. Biochem.*, 65, 445–448).
12 Benesch, R., MacDuff, G., and Benesch, R. E., *Analyt. Biochem.*, 11, 81–87 (1965).
13 Hill, A. V., *J. Physiol., Lond.*, 40, IV–VII (1910).
14 Wald, G., and Riggs, A., *J. gen. Physiol.*, 35, 45–53 (1951).
15 Briehl, R. W., *J. biol. Chem.*, 238, 2361–2366 (1963).
16 Benesch, R., and Benesch, R. E., *Science*, 185, 905–908 (1974).

Role of light and rhodopsin phosphorylation in control of permeability of retinal rod outer segment disks to Ca^{2+}

It has recently been shown in several laboratories that if isolated rod outer segments (ROS) are incubated in the presence of MgATP, rhodopsin is phosphorylated (see ref. 1). The reaction is much faster after the ROS have been exposed to light as the enzyme, 'opsin kinase', is specific to bleached rather than unbleached rhodopsin[1]. It has been suggested that the function of rhodopsin phosphorylation may be to regulate the responsiveness of ROS to light and in this paper we describe a mechanism whereby such a regulation could occur.

It is well established that the absorption of photons by
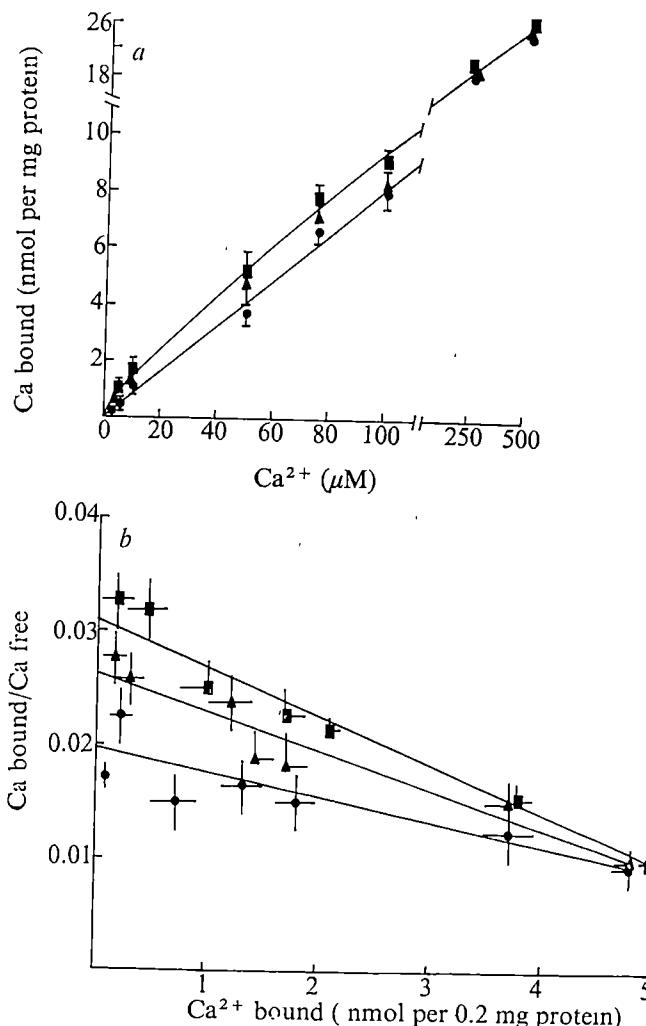
Fig. 1 Effect of Ca^{2+} concentration on the binding to ROS disks. Freshly prepared ROS were either kept in dim red light (●), exposed to white light for 2 min (▲), or exposed to white light for 2 min and phosphorylated at 37 °C at a protein concentration of about 0 5 mg ml^{-1} in 50 mM Tris-HCl, pH 7.4, and 1 mM MgATP for 30 min[1] (■). All samples were then washed by centrifugation from 10 mM Tris-HCl, pH 7.4, and incubated in dim red light at 37 °C at a protein concentration of about 0.2 mg ml^{-1} in 1 ml aliquots of 10 mM Tris-HCl, pH 7.4, and the stated concentration of $^{45}CaCl_2$ (specific radioactivity about 2×10^7 c p.m. μmol^{-1}) for 2 h to reach equilibrium (Fig. 3). Samples were then filtered using wet Millipore cellulose ester filters (0.45 μm pore size) and the filters washed with three 5 ml aliquots of 10 mM Tris-HCl, pH 7.4, placed in scintillation vials, and dried at 80 °C before adding scintillation fluid and counting. The washing procedure took less than 2 min and less than 2% of protein passed through the filters. Results are taken from five experiments and are shown as means ± s.d.

Fig. 2 Effect of exposure to light and phosphorylation on the rate of loss of Ca^{2+} from ROS disks. Freshly prepared ROS were either kept in dim red light (●), exposed to white light (▲), or exposed to white light and phosphorylated (■), and all samples washed with 10 mM Tris-HCl as described in Fig. 1. Samples were then incubated with 10 μM $^{45}CaCl_2$ (specific radioactivity 2×10^7 c.p.m. μmol^{-1}) in 0.3 M sucrose and 10 mM Tris-HCl, pH 7.4, at a protein concentration of about 0.3 mg ml^{-1} for 2 h and aliquots taken to determine the concentration of bound $^{45}Ca^{2+}$. The remaining material was centrifuged at 10^5g for 15 min and the pellets suspended in 10 mM Tris-HCl, pH 7.4, containing 0.3 M sucrose at a protein concentration of about 0.3 mg ml^{-1}. Samples (0.5 ml) were then taken at stated times and filtered through Millipore filters to determine the amount of $^{45}Ca^{2+}$ bound as described in Fig. 1. The time between the start of resuspension of the loaded pellets and the taking of the first sample was always 3 min. Results are taken from six experiments and show means ± s.d. The concentration of bound $^{45}Ca^{2+}$ at the end of the incubation with $^{45}CaCl_2$ was 1.78 ± 0.16, 1.90 ± 0.1 and 1.93 ± 0.1 nmol, $^{45}Ca^{2+}$ per mg protein for dark-kept, light-exposed, and light-exposed phosphorylated material, respectively.

vertebrate rods causes a hyperpolarisation which is thought to be the result of a decrease in Na^+ permeability of the ROS (refs 2–4). It is this hyperpolarisation which enables the photochemical apparatus of the outer segment to transmit its state as a nervous impulse. It is, however, difficult to see how the absorption of a single photon by the plasma membrane could cause a reduction in dark current by as much as 3% (ref. 5). Moreover most, if not all, of the light absorbing molecule, rhodopsin, is located not in the plasma membrane of the ROS but in the intracellular disks which are not connected directly to the outer plasma membrane[5,6]. These observations suggest that a transmitter is released from the ROS disks which interacts with the outer plasma membrane lowering its permeability to Na^+ (refs 3 and 7). Hagins[3,7] has suggested that the transmitter may be Ca^{2+} as treatment of ROS with Ca^{2+} causes their hyperpolarisation[7,8], and isolated ROS disks show an active uptake of Ca^{2+} (ref. 9). A light-induced efflux of calcium from ROS disks has also been described[10–13]. These observations indicate that even if Ca^{2+} is not the actual transmitter, it will have an important role in modulating the transmission of light as a nervous impulse.

There have been several reports that phosphorylation of plasma membrane proteins from liver[14], heart[15,16] or skeletal muscle[17] can increase the binding of Ca^{2+} to these membrane fragments. For this reason we investigated the effect of phosphorylation of rhodopsin on the Ca^{2+}-binding and permeability properties of ROS disks. /

ROS were prepared from calf eyes as described previously[18] and disk membranes and membrane fragments obtained by centrifugation at 10^5g for 60 min after rupturing the ROS outer

membrane by homogenisation in 10 mM Tris-HCl, pH 7.5 (ref. 19). The binding of Ca^{2+} to the disk membranes was determined after incubating to equilibrium as described in Fig. 1, from which it may be seen that there is little difference between membranes of ROS disks that have been kept in the dark, exposed to light or exposed to light and phosphorylated; although exposure to light and, in particular, phosphorylation of the light-exposed material, did cause some slight increase in Ca^{2+} binding. It has recently been reported that the amount of Ca^{2+} bound to ROS was greatly increased on exposure to light[12]. This result was probably obtained because incubations were not carried out for sufficient time to reach equilibrium (Fig. 3).

We next examined the effect of light on the rate of Ca^{2+} loss from ROS disks. Figure 2 shows that if the disk membranes were exposed to light before loading with $^{45}Ca^{2+}$ the subsequent efflux of radioactivity on suspension in Ca^{2+}-free medium was greater than that observed if the material was kept in the dark. The effect was prevented if the light-exposed material was phosphorylated by incubation with MgATP. Incubation with ATP in the absence of Mg^{2+} gave no effect. A similar effect of light was found whether the ROS disks were illuminated before or after loading with $^{45}Ca^{2+}$. As exposure to light causes, if anything, a slight increase in Ca^{2+} binding (Fig. 1) the increased $^{45}Ca^{2+}$ loss on exposure to light can scarcely be the result of an effect on binding; similarly phosphorylation causes such a small change in binding to light-exposed material that it is probably not important in affecting the rate of ^{45}Ca loss. It seems probable that Ca^{2+} entered the disks during incubation with $^{45}Ca^{2+}$ and that exposure to light increases calcium loss on suspension in Ca^{2+}-free media because of an increase in permeability of the disk membranes which makes it easier for Ca^{2+} to leave the loaded saccules, whereas phosphorylation of the light-exposed material lowers the membrane permeability.

If ROS disks which have been exposed to light are indeed more permeable to Ca^{2+} then the rate of entry of Ca^{2+} should also be increased. This was, in fact, found to be the case (Fig. 3); if the light-exposed material is phosphorylated then the rate of Ca^{2+} entry is lowered to a little less than that observed in material kept in the dark. Results shown in Figs 2 and 3 are thus comparable.

Reports have shown a decrease in bound Ca^{2+} on exposure of ROS to light[10–13], but it was not possible from those experiments to know if the result was the result of a reduction in

Fig. 3 Effect of light and phosphorylation on the rates of entry of Ca^{2+} into ROS disks. Freshly prepared ROS was either kept in dim red light (●), exposed to white light (▲), or exposed to white light and phosphorylated (■), and all samples washed with 10 mM Tris-HCl, pH 7.4, as described in Fig. 1. Disks were then suspended at a protein concentration of about 0.3 mg ml^{-1} in 10 mM Tris-HCl, pH 7.4, containing 0.3 M sucrose and 10 μM $^{45}CaCl_2$ (specific radioactivity 2×10^7 c.p.m. μmol^{-1}). Samples (0.5 ml) were taken at stated times and filtered to determine the amount of bound $^{45}Ca^{2+}$ as described in Fig. 1. Similar results have now been obtained in four separate experiments.
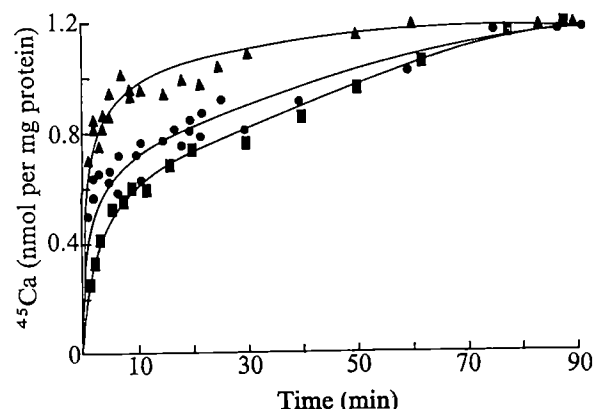
Ca^{2+}-binding sites or of a change in permeability. Our results clearly demonstrate that a change in permeability occurs.

If one accepts Hagin's hypothesis that a nervous impulse is triggered from ROS by the release of Ca^{2+} from the interdiskal space, then the observation that phosphorylation of bleached rhodopsin in the disks blocks the release is one of considerable importance. When light reaches the outer segments of the retina in the intact eye rhodopsin molecules will be bleached and a release of Ca^{2+} from the disks will certainly result. Within a fairly short time, however, bleached rhodopsin molecules will be phosphorylated and as this will lower Ca^{2+} permeability of the disks, they will be able to pump back Ca^{2+} from the extradiskal space. Such a mechanism would have considerable importance in the adaptation of retinal sensitivity to background light conditions. It is well known that the eye becomes progressively less sensitive with increasing background illumination; it seems likely that the fact that in such conditions increasing numbers of receptors are 'switched off' by phosphorylation will be of considerable importance in this phenomenon. On exposure to dark conditions the bleached rhodopsin will be regenerated and dephosphorylated so that the light receptors will be 'switched on' again, a fact which is likely to have considerable importance in dark adaptation. The time course of the loss of phosphate from phosphorylated rhodopsin in the intact frog retina is indeed the same as the time course of dark adaptation[20].

Plasma membrane fragments from many tissues, including brain[21-23], heart muscle[23,24], kidney[23] and liver[23], contain tightly bound kinase enzymes responsible for the phosphorylation of the membrane proteins. In these cases cyclic AMP stimulates the enzyme and has also been shown to cause an increase in efflux of Ca^{2+} from the kidney collecting tubule[25,26] or liver slice[27,28], and an increase of Ca^{2+} uptake into rat heart microsomal fragments[29,30]. Several workers have shown that phosphorylation of membrane proteins from liver[14], skeletal muscle[17] or heart muscle[15,16] causes an increase in Ca^{2+} binding, but no attempts have been made to measure possible changes in Ca^{2+} permeability. In the case of toad bladder, however, cyclic AMP causes an increase in the efflux of ^{45}Ca^{2+} presumably as a result of an increase in permeability[26,31]. In this tissue cyclic AMP stimulates the loss of phosphate from plasma membrane proteins, thus lowering the concentration of protein-bound phosphate[32]. Thus, in the toad bladder cell membrane, a decrease in protein-bound phosphate is associated with an increase in permeability to Ca^{2+}, whereas in ROS disks an increase in protein phosphorylation is associated with a decrease in permeability to Ca^{2+}.

It may be seen therefore that in the case of membranes of both ROS disks and toad bladder increased protein phosphorylation is associated with a decrease in permeability to Ca^{2+}. We suggest that changes in the state of protein phosphorylation have a general role in regulating Ca^{2+} permeability and/or binding in the cell membrane.

We thank G. Nullans for technical assistance and M.W. acknowledges receipt of a SRC fellowship.

MALCOLM WELLER
NOELLE VIRMAUX
P. MANDEL

Centre de Neurochimie,
11 rue Humann,
67085 Strasbourg CEDEX, France

1 Weller, M., Virmaux, N., and Mandel, P., *Proc. natn. Acad. Sci U.S.A.*, 72, 381–385 (1974).
2 Tomita, T., Miller, W. H., Hashimoto, Y., and Saito, T., *Expl Eye Res.*, 16, 327–341 (1973).
3 Hagins, W. A., *A. Rev. Biophys. Bioeng.*, 1, 131–158 (1972).
4 Korenbrot, J. I., *Expl Eye Res.*, 16, 343–355 (1973).
5 Penn, R. D., and Hagins, W. A., *Biophys. J.*, 12, 1073–1094 (1972).
6 Cohen, A. I., *Vision Res.*, 10, 445–453 (1970).
7 Yoshikami, S., and Hagins, W. A., in *Biochemistry and Physiology of the Visual Pigments* (edit. by Langer, H.), 246–255 (Springer, Berlin, 1973).
8 Hagins, W. A., and Yoshikami, S., *Expl Eye Res.*, 18, 299–305 (1974).
9 Bownds, D., Gordon-Walker, A., Gaide-Huguenin, A. D., and Robinson, W. E., *J. gen. Physiol.*, 58, 225–237 (1971).

10 Hendriks, Th., Daemen, F. J. M., and Bonting, S. L., *Biochim. biophys. Acta*, 345, 468–473 (1974).
11 Poo, M. M., and Cone, R. A., *Expl Eye Res.*, 17, 503–510 (1973).
12 Mason, W. T., Fager, R. S., and Abrahamson, E. W., *Nature*, 247, 562–563 (1974).
13 Szuts, E. Z., and Cone, R. A., *Fedn Proc.*, 33, 1471 (1974).
14 Shlatz, L., and Marinetti, G. V., *Biochem. biophys. Res Commun.*, 45, 41–50 (1971).
15 Will, H., Schirpke, B., and Wollenberger, A., *Acta Biol. med. Germ.*, 31K, 45–52 (1973).
16 Kirchberger, M. A., Tada, M., Repke, D. I., and Latz, A. M., *J. molec. cell. Cardiol*, 4, 673–680 (1972).
17 Sulakhe, P. V., and Drummond, G. I., *Archs Biochem Biophys.*, 161, 448–455 (1974)
18 Virmaux, N., Urban, P. F., and Waenheldt, T V., *FEBS Lett.*, 12, 325–328 (1971).
19 Meyer-Schultz, F., Porte, A., Nullans, G., and Virmaux, N., *Cytobiologie*, 7, 393–405 (1973).
20 Kuhn, H., *Nature*, 250, 588–590 (1974).
21 Weller, M., and Rodnight, R., *Nature*, 225, 187–188 (1970).
22 Weller, M., and Rodnight, R., *Biochem. J.*, 132, 483–492 (1973).
23 Rodnight, R., and Weller, M., in *Effects of Drugs on Cellular Control Mechanism* (edit. by Rabin, B. R., and Friedman, R. B.), 175–192 (Macmillan, London, 1972).
24 Krause, E. G., Will, H., Pelough, V., and Wollenberger, A., *Acta Biol. med. Germ.*, 31K, 37–43 (1973).
25 Aurbach, G. D., Marcus, R., Heersche, J, Marx, S., Niall, H., Tregear, C. W., Keutmann, H. T., and Potts, J. T., *Ann. N.Y. Acad. Sci.*, 185, 386–394 (1971).
26 Rasmussen, H, Goodman, D. B. P., and Tenenhouse, A., *Crit. Rev. Biochemistry*, 1, 95–148 (CRC, Cleveland, Ohio, 1972).
27 Friedman, N., and Park, C. R., *Proc. natn. Acad. Sci. U.S.A.*, 61, 504–508 (1968).
28 Friedman, N., *Fedn Proc.*, 30, 261 (1971).
29 Entman, M. L., Levey, G. L., and Epstein, S. E., *Circulation Res.*, 25, 429–438 (1969).
30 Shinebourne, E. A., and White, R. J., *Cardiovasc. Res.*, 4, 194–200 (1970).
31 Schwartz, I. L., and Walter, R., *Excerpta Medica Int. Congr. Ser.*, no. 16, 264 (1968).
32 Delorenzo, R., and Greengard, P., *Proc. natn. Acad. Sci. U.S.A.*, 70, 1831–1835 (1973).

Divalent cation ionophore A23187 forms lipid soluble complexes with leucine and other amino acids

THE *Streptomyces* antibiotic A23187, a divalent cation ionophore[1], has been used as a tool in studies of the role of calcium in several cellular phenomena[2-5]. A23187 has a much higher affinity for divalent than monovalent cations, and the increased permeability induced by this ionophore in cellular membranes favours calcium and magnesium. X537A, another divalent cation ionophore[6], also enhances fluxes of monovalent cations[7] and forms lipid-soluble complexes with noradrenaline and some other organic compounds[8]. A23187 has often been referred to as a specific divalent cation ionophore although, to our knowledge, the evidence for the specificity is restricted to the lack of interaction with common monovalent cations like sodium and potassium[1,2]. In this paper we report experiments which show that A23187 is able to form lipid-soluble complexes with leucine and other amino acids at concentrations as low as or lower than those needed to form similar complexes with calcium.

We have been studying the mitogenic effect of A23187 on human peripheral blood lymphocytes in culture (submitted for publication). During these studies we found that A23187, like the lectin mitogens[9], caused an enhancement of the rate of amino acid uptake by the cells within 3 h of culture. Unexpectedly, ethyleneglycol-*bis*-(aminoethylether)-tetra-acetic acid (EGTA) in concentrations which entirely blocked calcium uptake did not abolish the increased rate of amino acid uptake induced by the ionophore. We therefore examined possible direct interactions of the ionophore with leucine.

First, we investigated the effect of A23187 on the partitioning of L-leucine between an aqueous and an organic phase. ^{3}H-leucine and 10 μM A23187 in 1 ml phosphate-buffered saline (PBS) were shaken vigorously with the same volume of toluene–butanol (1:1 or 7:3 v/v) mixture[2,10]. The ionophore was able to increase the proportion of ^{3}H-leucine recovered in the organic phase by 5 to 20-fold (Table 1).

In preliminary experiments we found that some organophilic impurity was present in the ^{3}H-leucine preparation. Therefore, in later studies the ^{3}H-leucine solution was extracted once with the toluene–butanol mixture before using it in the

Table 1 Organic sequestration of ^{3}H-leucine by A23187

Composition of the initial water phase	Amount of leucine in the organic phase after extraction	
	% c.p.m.	nM
Experiment 1		
Leucine (18.5 nM)	2.34±0.02	0.43
Leucine (18.5 nM+A23187 (10µM)	11.87±0.25	2.19
Leucine (18.5 nM), pre-extracted	1.56±0.01	0.29
Leucine (18.5 nM), pre-extracted+		
A23187 (10 µM)	12.04±0.31	2.23
Experiment 2		
Cycle I:		
(A) Leucine (18.5 nM)	1.09±0.01	0.20
(B) Leucine (18.5 nM)+A23187 (10 µM)	19.76±1.85	3.65
(C) Leucine (18.5 nM)+ethanol (1%)	1.10±0.01	0.20
Cycle II:		
PBS only, shaken with the organic phase		
resulting from:		
Cycle IA	18.30±0.40	0.04
Cycle IB	23.55±0.25	0.86

[3]H-L-Leucine (1 µCi; 18.5 pmol); (Radiochemical Centre, Amersham, UK) in 1 ml PBS without divalent cations ± the ionophore as indicated was shaken with 1 ml toluene–butanol mixture for 1 min at room temperature in a conical glass centrifuge tube. Phase separation was facilitated by brief centrifugation. Aliquots of both the organic and the water phase were counted for radioactivity in identical conditions in a toluene-based scintillation cocktail. Total recovery of counts was more than 97%. Mean and range of the results from duplicate extractions are given. Approximate nM concentration of leucine in the final organic phase is calculated from the c.p.m. assuming that 100% of the radioactivity represents leucine.

Experiment 1: pre-extracted = ^{3}H-leucine stock solution extracted once with toluene–butanol before use in the actual experiment. Toluene–butanol ratio 1:1 v/v.

Experiment 2: Toluene–butanol ratio 7:3 v/v. % c.p.m. in cycle II is calculated with reference to the total amount of radioactivity in cycle II.

actual experiment. This procedure did not affect the A23187-induced sequestration of ^{3}H-leucine (Table 1, experiment 1). As the 10 mM stock solution of the ionophore was made up in ethanol, we included 1% ethanol in our controls. No significant increase in the proportion of ^{3}H-leucine in the organic phase was seen (Table 1, experiment 2). The A23187-induced sequestration of leucine was to a great extent reversible, as shown by re-extracting the organic phase with PBS (Table 1, experiment 2). As the vast majority of the ionophore is retained in the organic phase in these conditions[10], this finding suggests that some of the lipid-soluble complexes formed by leucine and the ionophore are dissociated during re-extraction with an aqueous phase. That the sequestration of ^{3}H-label by A23187 really represents transfer of leucine to the organic phase, and not that of a hypothetical radioactive impurity, was finally shown by displacing ^{3}H-label from the organic phase by unlabelled leucine (Table 2). These experiments also demonstrated that the molar ratio in the complexes formed by leucine and A23187 may be as high as 1:2, which is the figure reported for the divalent cation–A23187 complexes[3,10]. Ionophore (10 µM) was able to increase the concentration of leucine in the organic phase up to 6 µM (Table 2). Direct

measurements of leucine and ionophore concentrations are, however, needed to establish this ratio. Compared with cold leucine, $CaCl_2$ was clearly less effective in inhibiting sequestration of ^{3}H-leucine (Table 2). On the other hand, leucine seemed to be practically unable to affect the transfer of [45]Ca to the organic phase (Table 2). These findings could be explained by assuming that leucine and calcium are bound to different sites in the ionophore molecule, or that the $(A23187)_2$–Ca complex is not accessible to leucine, whereas complexes formed by the ionophore and leucine are sensitive to calcium ions. These competition experiments were complicated by the finding that 100 µM A23187, which was the concentration necessary to obtain significant sequestration of [45]Ca, was practically unable to transfer leucine to the organic phase, whereas such transfer was marked with the 10 µM concentration of the ionophore (Fig. 1). Almost identical results were obtained in three separate experiments. Higher concentration of ethanol in the 100 µM A23187 system was not the reason for the difference between the effects of 10 µM and 100 µM ionophore, as the presence of the same concentration of ethanol in the 10 µM system had no effect on sequestration of leucine or calcium (data not shown). We do not know the reason for the failure of 100 µM

Table 2 Competition between leucine and calcium for A23187

Composition of the initial water phase	% c.p.m. recovered in the organic phase	Organic sequestration % Inhibition	Calculated µM concentration of leucine or Ca in the final organic phase
[3]H-leucine (0.0185 µM)+A23187 (10 µM)			
+PBS	8.90±0.32	—	0.0016
+L-leucine (50 µM)	3.13±0.64	65	1.57
+L-leucine (500 µM)	1.22±0.06	86	6.10
+CaCl₂ (50 µM)	7.30±1.13	18	0.0013
+CaCl₂ (500 µM)	4.30±1.04	52	0.0008
[45]CaCl₂ (0.80 µM)+A23187 (100 µM)			
+PBS	14.7±0.9	—	0.12
+L-leucine (50 µM)	15.3±2.6	0	0.12
+L-leucine (500 µM)	13.9±0.5	5	0.11
+CaCl₂ (50 µM)	12.7±1.9	14	6.45
+CaCl₂ (500 µM)	5.1±1.2	65	25.54

Isotope (1 µCi; Radiochemical Centre) and the additions indicated were extracted with 1 ml toluene–butanol (7:3 v/v) mixture as described in Table 1. Approximate concentrations (µM) of leucine and calcium, respectively, in the final organic phase are calculated from the c.p.m., assuming that the affinity of the unlabelled compound for A23187 is identical to that of the respective radioactive isotope. Mean and range of the results from two separate extractions are given.

Table 3 Inhibition of ^{3}H-leucine sequestration by other amino acids and calcium

Composition of the initial water phase	Amount of ^{3}H-leucine in the organic phase after extraction		% Relative inhibition (mean)
	c.p.m.	μM (mean)	
^{3}H-leucine (50 μM)+A23187 (5 μM)	7,102±53	0.890	—
^{3}H-leucine (50 μM)+A23187 (5 μM)			
+L-leucine (50 μM)	5,678±322	0.710	100
+L-glycine (50 μM)	5,723±293	0.715	97
+L-lysine (50 μM)	5,832±122	0.725	89
+L-arginine (50 μM)	6,025±585	0.750	79
+L-aspartic acid (50 μM)	6,125±535	0.765	69
+L-glutamic acid (50 μM)	6,805±545	0.850	21
+β alanine (50 μM)	7,343±42	0.915	0
+ε amino caproic acid (50 μM)	7,458±178	0.930	0
+CaCl$_2$ (50 μM)	6,730±320	0.840	26

^{3}H-leucine (1 μCi) and the additions as indicated were extracted with 1 ml toluene–butanol mixture as described in Table 1. Relative inhibition of ^{3}H-sequestration by the added compounds is calculated from the c.p.m., taking the inhibition caused by 50 μM L-leucine as 100%. Mean and range of the results from two separate extractions are given.

A23187 to sequester leucine, but it could be explained by assuming that in an aqueous medium the highly hydrophobic A23187 can acquire two different micellar forms, depending on concentration. At lower concentrations of A23187 a form with a relatively high affinity to leucine is prevalent, whereas at higher concentrations the ionophore exists mainly in another form, which can bind calcium but not leucine. The final answer to this question requires further study.

We also found that leucine is not the only amino acid with which A23187 can interact. Whereas the partitioning of ^{3}H-uridine and ^{3}H-thymidine remained practically unaffected at 0.1 to 10 μM concentrations of the ionophore (data not shown) ^{35}S-methionine was effectively transferred to the organic phase by 10 μM A23187 (Fig. 1). Furthermore, several other α amino acids were able to inhibit organic sequestration of ^{3}H-leucine. They showed increasing relative affinity for the ionophore in the following order: Glu ≤ Asp < Arg < Lys < Gly < Leu (Table 3). Other experiments have shown that cysteine and glutamine can also inhibit sequestration of ^{3}H-leucine. Non-α

amino acids tested, β alanine and ε amino caproic acid, did not show any affinity for A23187 in this system (Table 3). Again, compared with cold leucine, CaCl$_2$ was less effective in inhibiting ^{3}H-leucine sequestration (Table 3).

The relevance of these findings to the effects of A23187 on cellular physiology is difficult to assess. Although we detected EGTA-resistant induction of an enhanced rate of leucine uptake within a few hours in lymphocyte cultures incubated with the ionophore, our attempts to show direct facilitation of leucine uptake in the presence of A23187 have so far been unsuccessful. Nevertheless, the findings reported in this paper indicate that the ionophore A23187 can cause reversible organic sequestration of amino acids at concentrations as low as, or lower, than those needed to form lipid-soluble complexes with calcium, and that equimolar concentration of calcium can only slightly inhibit this complex formation. This means that apart from the well documented action of A23187 as a potent divalent cation ionophore it may have effects on cellular metabolism resulting from its affinity for amino acids. One other implication of these findings is that when A23187 is used in complex experimental conditions, caution is necessary before concluding that all the phenomena detected are simply caused by increased permeability of membranes to divalent cations.

T. Hovi
S. C. Williams
A. C. Allison

Clinical Research Centre,
Watford Road,
Harrow HA1 3UJ, Middlesex, UK

1 Reed, P. W., *Fedn. Proc.*, 31, 432 (1972).
2 Reed, P. W., and Lardy, H. A., *J. biol. Chem.*, 247, 6970 (1972)
3 Foreman, J. C, Mongar, J. L., and Gomperts, B D., *Nature*, 245, 249 (1973).
4 Steinhard, R. A., and Epel, D, *Proc. natn. Acad Sci. U S A*, 71, 1915 (1974).
5 Maino, V. C., Green, N. M., and Crumpton, M. J, *Nature*, 251, 324 (1974).
6 Entman, M. L, Gillette, P. C., Wallick, E. T, Pressmann, B. C, and Schwartz, A., *Biochem. biophys. Res. Commun.*, 48, 847 (1972)
7 Pressmann, B C, and de Guzman, N T., *Ann. N.Y. Acad. Sci.*, 227, 380 (1974)
8 Pressmann, B. C., *Fedn. Proc*, 32, 1968 (1973).
9 Van den Berg, K J, and Betel, I., *Expl Cell. Res*, 66, 257 (1971).
10 Pfeiffer, D. R., Reed, P. W., and Lardy, H. A, *Biochemistry*, 13, 4007 (1974).
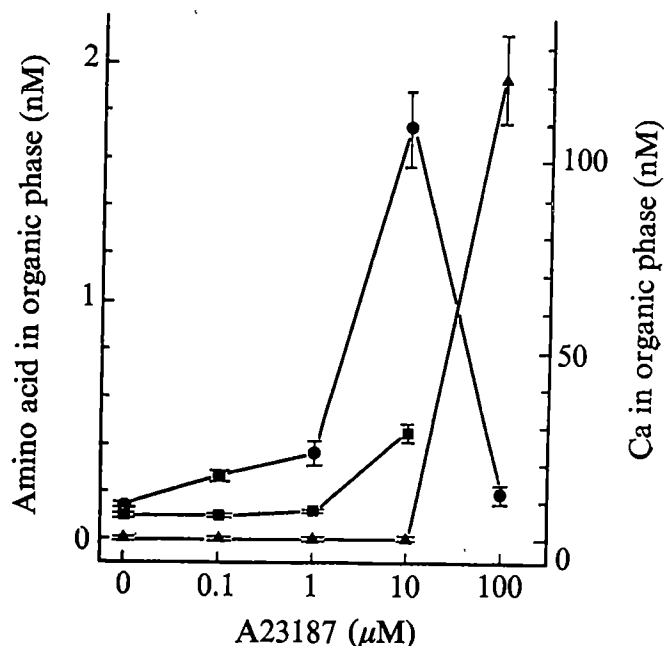
Fig. 1 Dose dependence of sequestration of ^{3}H-leucine, ^{35}S-methionine and ^{45}Ca into organic phase by A23187. Radioisotope (1 μCi) and indicated concentrations of the ionophore in 1 ml PBS were mixed with 1 ml toluene–butanol (1:1 v/v) mixture and shaken vigorously for 1 min at room temperature. Initial concentrations in the water phase were: ^{3}H-leucine, 18.5 nM; ^{35}S-methionine, 8.0 nM; ^{45}CaCl$_2$, 800 nM. For other experimental details see Table 1. Mean ± s.d. of two to four experiments are given. ●, Leucine; ■, methionine; ▲, calcium.

Corrigendum

In the article "The function of phytochrome in plants growing in the natural environment" by M. G. Holmes and H. Smith (*Nature*, 254, 512; 1975) the two arable weeds used were *Tripleurospermum martimum inodorum* (scentless mayweed) and *Chenopodium album* (fat-hen).

reviews

HERE is something to hold on to when exploring the complex terrain of animal learning. Professor Mackintosh has given us a book* that is detailed, authoritative and scholarly. In this age of symposia and multiauthored volumes, it is a rare treat to find a work of this magnitude by a single author, and even rarer to find one with an incisive and economical style. It is a large book, with more than 600 pages of text and over 2,000 references. It is also often a tough book, not only because its author does not hesitate to deal with complicated issues and material, but because his very economy of style does not cushion the reader with redundancy. It is a book to read without blinking, if not unblinkered. More than that : it is to be reread and kept as a source of relevant literature and of perceptive commentary and sophisticated analysis. Both the student and his mentor will be grateful.

The subject matter is conventional and traditional to experimental psychology—at least, it used to be traditional. It deliberately avoids the ethologists' concern with differences in creatures' capacities forced on them by particular evolutionary pressures "Although there may be important differences in the processes of behavioural modification to be found in different animals, the study of those processes, independently of any particular concern for their specialisation in different groups, remains an important and valid branch of science. Just as the sciences of genetics, embryology, and neurophysiology were initially advanced by the search for general, fundamental principles, undertaken with subjects chosen solely for reasons of convenience, so it is reasonable to suppose that some principles will have emerged from the analysis of associative learning in dogs, rats and pigeons. It is, at any rate, this tradition, started by Thorndike in America and, more systematically, by Pavlov in Russia, that forms the subject matter of this book." (p.3).

As such, a large portion of the volume concentrates on the details and theoretical underpinning of Pavlovian and instrumental conditioning in the dog, rat and pigeon. The first really influential book that attempted to contrast and dissect these two learning

*The Psychology of Animal Learning. By N. J. Mackintosh. Pp. xiv+730. (Academic : London and New York, December 1974.) £8.00; $18.50.

paradigms was Conditioning and Learning, by Hilgard and Marquis in 1940, and the comparison with the present work is of some interest. The empirical evidence now has swollen, almost, some would say, to the point of diminishing returns in a few areas of concentration. Not surprisingly, it has also become fragmented and there has resulted, Mackintosh fairly points out, "a severe failure of communication between workers in closely related areas. Thus [for example] the analysis of reinforcement in classical condition-

Careful tread on classic ground

L. Weiskrantz

ing has had little impact on theories of instrumental learning "

Mackintosh goes a long way towards re-establishing these lines of communication, and he can draw on recent advances that, somewhat overdue, have flowed from the liberation of behavioural analysis from the apparatus and paradigms that in the past have been taken almost as definitions of a particular type of learning. Thus, the discovery of autoshaping has planted a classical conditioning colony right in the middle of the instrumental conditioning reservation. Further, almost wholly new areas of interest have emerged; for example, contrast effects earn a whole chapter in Mackintosh's book whereas the term was not even known to Hilgard and Marquis, and in general the findings and experimental niceties of the operant conditioning movement are seen throughout this volume. Significantly, Hilgard and Marquis devoted hardly any space to the analysis of punishment, either theoretical or empirical, and Mackintosh's account of this important area is especially useful and succinct. Finally, it is interesting that Mackintosh—in a much stouter volume—foregoes discussion of some of the final chapter headings of Hilgard and Marquis—for example, neurophysiological mechanism and personality (both of deep concern to Pavlov)—and only touches briefly on the question of problem solving. One suspects that it

was not solely for reasons of limitations of space that this self-restraint was exercised.

It is fair to ask whether new principles have, in fact, emerged from persisting with the traditional account of associative learning in animals, as Mackintosh thinks it reasonable to suppose will have happened. Certainly, many new assertions about limited domains have emerged, but the striking strength of this book is its disavowal of easy generalisations; it takes complexity seriously, and abounds with qualifications and counter-arguments, laced with scepticism. Talking about schedules of reinforcement, a prime source of generalisations for some researchers, he says "They are frighteningly complex and not necessarily at all well suited to an elucidation of the important processes underlying instrumental behaviour. Too many variables are permitted to vary in too many ways for it to be easy to achieve any analytic understanding." At the same time, he is prepared to defend unpopular theories, such as the stimulus-substitution theory of classical conditioning. Elsewhere he is at pains to blur accepted distinctions, as when he argues that the difference between response systems of classical and instrumental conditioning is one of degree, not of kind. Overall, the case for dealing with associative learning in animals, independent of particular specialisations, is well sustained, though whatever else this volume provides, it is not a compendium of fundamental principles. But progress in this field need not suffer on that account. In some other areas of biology, such as neurophysiology, we continue to see significant advances based on the gradual accumulation of evidence combined with careful empirical and logical dissection of the controlling variables and the underlying mechanisms, rather than the all-embracing insight or sudden breakthrough.

Beyond the domain of conditioning and the excellent extensions into discrimination learning, contrast, and generalisation, one will not find much argument or space devoted to cognitive capacities commonly appealed to in studies of human learning that might also seem applicable to learning in other mammals. Even place learning, long a favourite of the animal 'cognitive' theorists, is relegated to minor importance. Learning-set phenomena are discussed only briefly. New fron-

tiers may not be broached, but a large
and strategically located segment of the
Old World has been extremely well
charted. One will have to look hard
for a better guide to a region that is
criss-crossed with well trodden paths
but still nourishes a fresh crop of deli-
cate fruit and nettles each spring. For
someone who wants to keep his feet on
the ground, nettles really matter. □

Simply chemistry

Chemistry. By Linus and Peter Paul-
ing. Pp. xi+767. (Freeman: San
Francisco, 1975.) $13.95.

THIS is essentially a new edition of the
elder Pauling's highly successful
General Chemistry. The original text
has been shortened in places, largely by
the omission of much of the thermo-
dynamic material, and the scope has
been expanded by the addition of an
extensive and well balanced treatment
of molecular biology, in which most of
the important macromolecular struc-
tures are discussed. This change reflects
not only some shift in the research
emphasis of chemistry itself but also in
the new audience for whom this book
has been specifically designed, namely
"students primarily interested in bio-
logy, medicine, human nutrition, and
related fields". It provides thus a broad-
brush picture of the whole of chemistry
(inorganic, physical, nuclear, organic,
and biochemistry) and its interaction
with the biological sciences, at a level
suitable for those first studying it at an
American college.

The emphasis throughout is on the
structural aspects of chemistry, even
more so than in the previous editions.
For many chemists the chief demerit
of *Chemistry* will be its heavy reliance
on electronegativity, resonance, the
electroneutrality principle, and other
concepts familiar to readers of *The
Nature of the Chemical Bond* but less
obvious in most of the current che-
mical literature: the authors would
presumably defend their point of view
as being simple, general, and par-
ticularly suited to the limited chemical
sophistication of their intended readers.
Others may cavil at the slight reference
to modern physical techniques and
organic reactions and their mechanisms.
The exposition, which is delightful
with its clarity and elegance of style,
will appeal to the student and arouse
his or her interest with its neat and
modern choice of example and illustra-
tion. There are numerous sensible,
straightforward, and intriguing prob-
lems and the diagrams are excellent. I
much enjoyed reading it, and so did
my daughter, now in the middle of a
sixth form science course. By modern
standards the book is very modestly
priced. **C. S. G. Phillips**

From cell to cell

Cell Communication. (Wiley Series in
the Dynamics of Cell Biology.) Edited
by R. P. Cox. Pp. ix+262. (Wiley: New
York and London, November 1974.)
£11.90.

ONCE again we are victims of editorial,
or perhaps publisher's pride—this book
is not about all aspects of cell com-
munication for it treats only a limited
number of features of the subject. But
there is one strong, important theme of
the book: it contains three good re-
views on the related subjects of 'tight
and gap junctions', 'low-resistance path-
ways' and 'metabolic cooperation'. The
remaining eight papers cover an assort-
ment of subjects in which cell com-
munication plays a greater or lesser
role but no particular theme joins them
or excuses the absence of so many
aspects of cell communication. The
diligent reader will note the lack of any
mention of exo and endocytosis, cell
fusion (both in fertilisation and in other
contexts), cell adhesion, cell positioning
and recognition phenomena, malig-
nancy, and anything at all about plants.

The electron microscopists seem at
last to have abandoned their addiction
to neoclassical microanatomical latinity
and the excellent article by Norton
Gilula shows how *zonulae occludentes*
have become tight junctions. But this
is not the main virtue of his article.
That lies in the beautiful electron
micrographs of the specialised contacts
between cells which are probably the
low-resistance routes between cells and
the pathways for interchange of
metabolites between cells. This article
summarises work from several labora-
tories, published in the past few years.
Judson Sheridan explains the main
observations carried out on low-
resistance pathways and the editor and
his coworkers review "Metabolic co-
operation". In spite of the suggestion
that appears in these three articles—
that the three phenomena are but
aspects of the same matter—it is still
unclear as to how important the
phenomena are in the real life of an
animal.

The remaining articles in the book
cover an assortment of subjects. Note-
worthy are those by Harry Rubin on
cell growth—a process in which cell
communication may be unimportant—
and by Albert Harris on contact in-
hibition. There are also papers by
Fishbach on neuromuscular junction
formation, Kolodny on transfer of
macromolecules between cells, Cruik-
shank on cell interactions in the skin,
Basten and Miller on the immune
response, Neufeld on lysosomal diseases
and Ottolenghi-Nightingale on DNA
transformation in mammalian cells.
Adam Curtis

ABOUT a century ago science was in the grip of a surging interest in psychical research. In laboratories, both amateur and professional, literally hundreds of earnest scientists were conducting experiments of the most extraordinary kind—bottling ectoplasm, weighing mediums while in and out of trance, photographing materialised spirit forms, and so on. Though it may seem that such activities were at serious odds with the 19th century mechanistic view of the Universe, on second thought they seem less incongruous. Most of these pioneers were raised and educated within the ethos of Victorian Christianity which held that man was essentially an immortal spirit for whom death was a transition and not an extinction point. Furthermore, they were also steeped in the notion of the infallibility of the 19th century scientific method—everything in the Universe was fit and ready for instant laboratory investigation. Thus the weird phenomena of psychic research were no more elusive in principle than the behaviour of molecules or planets.

The rollcall of personalities involved in that phase of psychical research is breathtaking and includes Lodge, Rayleigh, Freud, Wallace, Richet, Crookes, Gladstone and J. J. Thomson, to name but a few; all came to believe that psychic research was one of the most important avenues of study for mankind, and incurred the contemptuous scorn of fellow academics as a result. Sir William Crookes for example, one of the most distinguished physicists of the century, participated actively in spiritualist seances and was so confident of his beliefs that he proudly allowed himself to be photographed arm-in-arm with a "materialised spirit form", a diaphanously garbed but otherwise rather earthy-looking being, known as Katie King.

Well, a 100 years have rolled by and, in spite of the sonorous declarations of Crookes and his contemporaries and the volumes of scientific papers on psychical research, who today—apart from a few ardent spiritualists—treats bottled ectoplasm or the photographs of the Crookes-Katie King encounter as anything other than fading curiosities? Worse still, who would argue that all the industry of those gallant pioneers and the stupendous cerebral effort they invested in chasing phantoms has improved our understanding of the Universe one jot?

I have to admit that disheartening reflections of this kind drifted through my head as I scanned through John Taylor's *Superminds**. The author is a

*Superminds: An Enquiry into the Paranormal. By John Taylor. Pp. 183. (Macmillan: London and Basingstoke, April 1975.) £3.95.

distinguished mathematician and physicist with an international reputation in his field. He is also at this time actively preoccupied with investigating phenomena which, if not actually involving ectoplasm and spirits appearing out of floors, will seem to most scientists to be more or less in the same category. The phenomena in question involve mysterious distortions in objects such as spoons, forks and metal bars, which are alleged to take place in the general vicinity of certain children. I say "in the general vicinity of" and yet, though most of the bendings and distortions —some of them are literally grotesque, as the book's numerous illustrations reveal—require the child to stroke or

Brave but not convincing

Christopher Evans
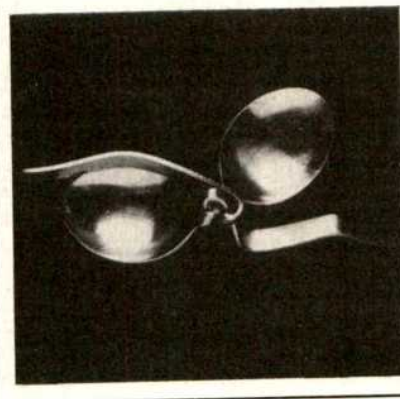
otherwise handle the metal, others allegedly take place at distances varying from yards to miles away from the 'bender'. As if that were not enough, the objects sometimes bend inside metal boxes, corked test tubes and even in locked cupboards.

Professor Taylor's interest in this peculiar pocket of the Universe seems to have been sparked by the notorious BBC television programme in which the flamboyant Uri Geller disintegrated a fork before an historically uncritical audience. This was followed by more closely controlled demonstrations in Taylor's laboratory at King's College, some of which unsettled him to the extent that, as he puts it, "the whole framework with which I viewed the world had suddenly been destroyed". From here there seemed no course but to continue the investigations through to the end, whether bitter or glorious, taking as subjects some of the children whose metal bending powers sprang into being following the television demonstration.

As a result Taylor is convinced that he is tapping and probing previously unknown forces of immense potential and significance, and his *Superminds* is in part a record of his findings and in

part an attempt at providing a theoretical framework for them. For the latter he leans on hypotheses invoking electromagnetism, mainly because no other kind of theory even remotely begins to fit the facts. The soundness of this theorising is a matter for discussion by other theoretical physicists but I must raise the strong suspicion that Professor Taylor is putting the cart before the horse. Before getting drawn into the complexities of theorising it is more economic to make absolutely certain that one has not only established the phenomena beyond all doubt, but that one has also satisfied oneself that these phenomena cannot be encompassed within any existing theory.

Unfortunately, though Taylor may well be convinced of this in his own mind, the material in the book will, I fear, do little to convince other scientists. My most serious objection is the ease with which he seems able to discount the possibility of fraud and deception; true, he has devised some elaborate apparatus—attaching the objects to spring balances to detect whether the benders are using physical pressure on them, and so on—but these precautions seem to me to be diversionary and indirect when more positive strategies could be used.

For example, if it is claimed that an individual can bend metal, within containers of various kinds, without touching it, then the first step should be to set up apparatus in an independent laboratory, using infrared beams to detect prying fingers, and videotape to record the metal as it distorts. Alternatively, if it is argued that laboratory conditions are inhibiting—Taylor comments, rather ingenuously, that the presence of "sceptics" tends to kill the phenomena—then all one has to do is to encapsulate metal bars in specially blown glass spheres which can be left for a given period in the homes of the 'superminds', and which can be inspected afterwards for signs of tampering. In fact, since writing *Superminds* Taylor has been attempting automation of his experiments, including constant videotaping, in an attempt to meet objections of this kind. The truth of the matter is, however, that if half of what is claimed on behalf of the superminds is true, then scientists and science in general deserve more solid data than he offers in this particular work. But it is still a brave book and Professor Taylor is a brave man to have written it. Alas it is the same kind of bravery, in my view, that Crookes exhibited when he took an alluring ghost by the arm and tried to convince the world of her reality. Crookes' contemporaries remained unconvinced, and so, I suspect, will Taylor's. □

obituary

Ernst F. W. Alexanderson died on May 14 at Schenectady, New York, where for so many years he was a rich source of ideas and inventions for General Electric. He was 97.

General Electric, USA

Alexanderson was born at Uppsala, Sweden and studied at the University of Lund and the Royal Institute of Technology in Stockholm. Shortly after graduating he was working in Germany when he saw a paper on 'Alternating Current Phenomena' by Steinmetz and decided to go to the United States and seek employment in the author's laboratory at General Electric. He went to America in 1901, started work under Steinmetz in 1902, and within a year had established a reputation as a designer specialising in alternating current generators. At this time, just after the turn of the century, commercial wireless telegraphy was well established, but the system most generally used was the fairly crude spark transmission, which radiated a damped wave train covering an inconveniently wide frequency band and which was unsuited to any modulation more sophisticated than the on–off keying of the Morse code. If a continuous, high frequency, sinusoidal voltage could be generated it would help solve the increasingly pressing problem of interference between simultaneous transmissions from neighbouring stations, and furthermore it would be a much more promising carrier for

modulation by speech or music. In 1904, Fessenden, who had carried out successful preliminary experiments with radio telephony, brought this problem to General Electric and asked them to design a high power alternator operating at a frequency of many kHz. The task was given to Alexanderson, and on Christmas Eve, 1906, Fessenden was able to broadcast speech and music. Work on thermionic valves proceeding elsewhere in the General Electric laboratories led to efficient methods of high power modulation, and Alexanderson's alternators, producing up to 200 kW, became the basis of many commercial and military radio systems during World War I. This strongly influenced the US Government to promote the formation in 1919 of the Radio Corporation of America, combining the radio interests of General Electric with those taken over from foreign companies who were excluded from the new organisation in the national interest. Alexanderson worked full-time for General Electric and RCA until he was seventy, and as a consultant until he was nearly eighty, pouring out inventions in fields as diverse as magnetic amplification, control systems, ship propulsion, aerial design, electronic circuitry and colour television systems. He was in the great tradition of American inventor–scientists and had well over three hundred patents to his name. Of his many honours, he was probably most gratified by a decoration from King Gustav V of Sweden and by the entirely appropriate award of the Edison medal.

G. K. T. Conn, OBE, professor of physics and head of the department in the university of Exeter since 1957, died on June 4 at the age of 64

After working on infrared and Raman vibrational spectra with Sutherland's group in Cambridge, he worked both at Sheffield and Exeter on the properties of solids (metals, alloys and semiconductors)—as determined by the evaluation of the optical constants for various frequencies. This involved determining the parameters of monochromatic radiation reflected from prepared surfaces; and the frequencies involved ultraviolet, visible, infrared and far infrared, including latterly the methods of Fourier transform spectroscopy. The methods he developed with Beattie and Eaton have been adopted by a number of other workers in the

field. He was editor of *Research*, a review journal, from 1956–62, and more recently had been joint editor of a series of Essays in Physics. He was also general editor of a series of textbooks, *The Modern University Physics Series*, in current production. His terms as deputy vice-chancellor of Exeter (1967–69) involved him in a series of delicate discussions during a period of unrest besetting British universities.

Hans D. Berendes, professor of genetics at the University of Nijmegen and well known for his work on gene action and chromosome structure, died on May 25 at the age of 41.

Berendes was born in The Hague and in 1955 began his study in biology at the University of Leiden. In the advanced part of his study he started research on the salivary chromosomes of *Drosophila hydei*, which dominated his whole scientific career, and completed a cytological map of these giant chromosomes. In 1962 he was appointed as lecturer at the University of Leiden. Impressed by the work of Beermann and Clever he worked on gene action in *D. hydei* as expressed in the puffing of salivary chromosome bands. He obtained his doctors degree in 1965 for a thesis on puffing patterns in the course of the late development of larvae and pupae, and the correlation between the appearance of some puffs and a cell product. In the same period he started research on the induction of puffs by external factors, in particular heatshocks. He then took a position in Beermann's laboratory at Tübingen, where he worked on the role of ecdyson in activation of puffs, puffing patterns in different tissues, protein metabolism in puffs, differential replication of chromosomal DNA and the electron microscopic structure of the salivary chromosome. In 1968 the Universities of Geneva and Nijmegen offered him chairs in Zoology, but he preferred to return to Holland to continue his work on *D. hydei*. Important papers appeared on the ultrastructure of DNA from the giant chromosomes, on the products of certain puffs, and on the relation between the formation of puffs and synthesis of mitochondrial enzymes. The latter project promises important insight into the regulation of gene activity in higher organisms. Berendes was also a very able organiser. He participated strongly in University affairs and in the Organisation for Pure Research (ZWO).

announcements

Award

Lynn R. Sykes has been awarded the **Walter H. Bucher Medal** by the American Geophysical Union for original contributions to the basic knowledge of the Earth's crust.

Appointments

P. J. Peterson has been appointed Royal Society visiting professor attached to the School of Biological Sciences at the Universiti Sains Malaysia.

James Brown, area engineer for Africa and the Western Hemisphere, Shell International, has been appointed to the new chair of petroleum engineering at Heriot-Watt University.

The University of Sheffield has made the following appointments: **N. M. Atherton** to a personal chair in chemistry; **G. Hudson** to a personal chair in experimental haematology.

Miscellaneous

Heinz Karger Prize. Heinz Karger Memorial Foundation invites papers on: methods for early diagnosis of genetic disorders (1976); and molecular biology of metabolic diseases (1977). Closing dates: February 28, 1976 and 1977. For conditions and further information, contact: S Karger AG, Arnold-Bocklin-Strasse 25, CH-4011 Basle, Switzerland.

Travel fellowships. The Winston Churchill Memorial Trust offers travel grants for 1976. This year's list of categories includes: teachers in further education, laboratory technicians, energy resources, naturalists, and conservation of coastal amenities. Further information and applications (UK citizens only): The Winston Churchill Memorial Trust, 15 Queen's Gate Terrace, London SW7 5PR, UK.

International meetings

August 21–23, Gamete competition in plants and animals, Lake Como, Italy (Dr D. Mulcahy, Universita di Milano, Istituto di Genetica, Via Celoria, 10, 20133 Milano, Italy)

August 21–27, Plant protection, Moscow, USSR (Professor V. A. Lebedev, Secretary General of the Organising Committee of the Seventh International Congress of Plant Protection, Orlikov per 1/11, Room 478, Moscow 107139, USSR).

August 22–29, Limnology, Winnipeg, Manitoba (Mr R. M. Raeburn, University Relations and Information Officer, The University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada).

August 23–27, Rheology, Gothenburg, Sweden (J. Kubat, Chairman, Organising Committee, Chalmers University of Technology, Fack, S–402 20, Goteborg 5, Sweden).

August 24–27, Crystal growth, Edinburgh, UK (F. W. Ainger, Allen Clark Research Centre, The Plessey Company Limited, Caswell, Towcester, Northamptonshire, UK).

August 25–29, Atomic spectroscopy, Clayton, Australia (Dr J. B. Willis, Secretary, Fifth International Conference on Atomic Spectroscopy, CSIRO Division of Chemical Physics, PO Box 160, Clayton, Victoria 3168, Australia).

August 25–29, Thin films, Budapest, Hungary (Organising Committee of ICT F3, 1325 Budapest, PO Box 76, Hungary)

August 25–29, Cybernetics and systems, Bucharest, Rumania (Dr J. Rose, Director General, World Organisation of General Systems and Cybernetics (WOGSC), College of Technology, Blackburn BB2 1LH, UK).

August 26–29, Antivirals with clinical potential, Stanford, California (Antivirals Symposium, Division of Infectious Diseases, Stanford University School of Medicine, Stanford, California 94305).

August 27–30, Phonon scattering in solids, Nottingham, UK (Phonon Conference Department of Physics University of Nottingham, Nottingham NG7 2RD, UK).

August 28–30, New first and second messengers in nervous tissues, Brescia, Italy (Dr M Trabucchi, Scientific secretary, Institute of Pharmacology and Pharmacognosy, University of Milan, Via Andrea Del Sarto, 21, 20129, Milan, Italy).

August 29–31, Function and metabolism of phospholipids in nervous tissue and **Biochemical and pharmacological implications of ganglioside functions**, Perugia, Italy (Professor G. Porcellati, Chairman, Organising Committee, Instituto di Chimica Biollogica, Universita di Perugia, Policlinico Monteluce, C.P. 3, Succ.3, Perugia, Italy).

August 31–September 5, Charles Lyell centenary symposium, London, UK (J. C. Thackray, Geological Museum, Institute of Geological Sciences, Exhibition Road, London SW7 2DE, UK).

Reports and publications

Great Britain

Proceedings of the Royal Irish Academy. Vol. 75, Section A. No. 5: Circumscribing Quadrics—a New Approach to the Problem of Statistical Discrimination. By I G. O. Muircheartaigh. Pp.49-56. 24p. No. 6: The Nature of the Vortex Core. By P. D McCormack. Pp.49-56. 24p. No. 6: The Nature of the Vortex Core. By P. D. McCormack. Pp 57-72. 30p. No. 7. Properties of the Variation. By P. McGill. Pp.73-78. 18p. No. 8: SL(2,C) and the Lorentz Group. By J. J. McMahon. Pp.79-83 18p. Vol. 74, Section B. No. 4: Records of Codium Species in Ireland. By Hilda M. Parkes. Pp.125-134. 35p. No. 5: Sorption of Carbon Dioxide. and Ammonia by Zeolites Containing Univalent and Trivalent Cations. By B. Coughlan and S. Kilmartin. Pp.135-154. 45p. No. 6. The Old Red Sandstone Group of Iveragh, Co. Kerry. By J. G. Capewell. Pp.153-171. 37p. No. 7. Deep-Seated Igneous Intrusions in Co. Kerry. By D. W. Howard. Pp.173-183+plates 2 and 3. 46p. No. 8 Contributions to the Lichen Flora of South-East Ireland—I. By M. R. D. Seaward. Pp.185-205. 34p. No. 9: The Pattern of Glaciation of County Dublin. By P. G. Hoare. Pp.207-224. 48p. (Dublin: Royal Irish Academy, 1975.) [154

The Achievement of Television. By Huw Wheldon. (BBC Lunch-Time Lectures, Ninth Series, 5.) Pp.18. (London: BBC, 1975.) [164

The Institute of Fuel. Report and Accounts for 1974. Pp 12. (London: The Institute of Fuel, 1975.) [164

Forestry Commission. Report on Forest Research 1974. Pp.vii+109+4 plates. £1.10 net. Fifty-Fourth Annual Report and Accounts, 1973/1974. Pp.102+6 plates £1.05 net. (London: HMSO, 1974.) [184

Memoirs of the Royal Astronomical Society, Vol. 78, Part 3. Aystematic Errors in the Velocities of Galaxies. By R. M Lewis Pp.75-100. (Oxford and London: Blackwell Scientific Publications, 1975. Published for the Royal Astronomical Society). [184

Bulletin of the British Museum (Natural History). Botany. Vol. 5, No. 3: The Marine Algae of Trinidad, West Indies. By W. D. Richardson. Pp.71-143+12 plates. £5.20. Entomology. Vol. 32, No. 1: A Revision of the African Ponerine Ant Genus Psalidomyrmex Andre (Hymenoptera·Formicidae). By B. Bolton. Pp.1-16 £1.30. Vol. 32, No. 2: The Butterflies Named by J. F. Gmelin (Lepidoptera: Rhopalocera). By R. I. Vane-Wright. Pp.17-64+6 plates. £3. Supplement 25: A Reclassification of the Arctiidae and Ctenuchidae Formerly Placed in the Thyretid Genus Automolis, with Notes on Warning Coloration and Sound. By A. Watson. Pp 104+34 plates. £9.75. (London: British Museum (Natural History), 1975.) [184

The Zoological Record. 1970, Vol. 107, Section 6: Vermes, Part A Complied by Eileen Mitchell and R. A. Bray. Pp.vi+175. £6 65. 1971, Vol. 108, Section 4: Coelenterata. Compiled by the Staff of the Zoological Society of London Pp.vii+62. £6. 1971, Vol. 108, Section 6: Vermes, Part C. Compiled by the Staff of the Zoological Society of London. Pp.vi+9. £3.75. 1971, Vol. 108, Section 8: Bryozoa (Polyzoa) and Entoprocta. Compiled by the Staff of the Zoological Society of London. Pp.vi+19. £3.75. 1971, Vol. 108, Section 9: Mollusca. Compiled by the Staff of the Zoological Society of London. Pp.viii+277. £10. 1971, Vol. 108, Section 12: Arachnida. Compiled by the Staff of the Zoological Society of London. Pp.vii+119. £7.50. 1971, Vol. 108, Section 14: Protochordata etc. Compiled by the Staff of the Zoological Society of London. Pp viii+27 £6. (London: The Zoological Society of London, 1975.) [184

Forestry Commission. Research and Development Papers. No. 108: Tree Growth on the South Wales Coalfield. By G. J. Mayhead, K. Broad and P. Marsh. Pp.iii+27. No. 109 Fertiliser Effects on the Growth and Composition of Sitka and Norway Spruce Nursery Transplants and on the Composition of a Podzol Profile After 15 Years Cropping. By Blanche Benzian, J. Bolton and J. K. Coulter. Pp.14. No. 110: Initial Spacing in Relation to Establishment and Early Growth of Conifer Plantations. By A J. Low. Pp.14. (London: Forestry Commission, 1974.) [184

The Ciba Foundation for the Promotion of International Co-operation in Medical and Chemical Research. Annual Report 1974. Pp.54. (London. The Ciba Foundation, 1975.) [214

Mathilda and Terence Kennedy Institute of Rheumatology. Eighth Annual Report, 1974. Pp.76, (London. Mathilda and Terence Kennedy Institute of Rheumatology, 1975.) [214

British Mesozoic Fossils. Fifth edition. Pp.vi+207 (73 plates). (London· British Museum (Natural History), 1975.) [224

A Key to the Larvae, Pupae and Adults of the British Dixidae (Diptera), The Meniscus Midges. By Dr. R. H. L. Disney· Pp.78. (Ambleside, Westmorland: Freshwater Biological Association, 1975.) £1. [224

Mineral Resources Consultative Committee. Mineral Dossier No. 11. Ball Clay. Compiled by D. E. Highley. Pp.v+31. (London: HMSO, 1975.) 61p net. [244

Writing a Scientific Paper. By Vernon Booth. Third edition. Pp.26. (London: The Biochemical Society, 1975. Published by permission of Koch-Light Laboratories, Ltd.) 50p; $1.50. [244

Philosophical Transactions of the Royal Society of London. A: Mathematical and Physical Sciences. Vol. 278, No. 1282 The Magnetic Transition on Moderately Small Superconductors, and the Influence of Elastic Strain. By B. Rothberg Bibby, F. R. N. Nabarro, D. S. McLachlan and M. J. Stephen. A Possible Hypo-Critical Point in the Phase Diagram of a Moderately Small Superconductor in a Magnetic Field. By F. R. N. Nabarro, and B. Rothberg Bibby. Pp.311-349. UK £1.60; Overseas £1.65. Vol. 278, No. 1283: Studies in Hydrodynamic Thrust Bearings. I. Theory Considering Thermal and Elastic Distortions. II. Comparison of Calculated and Measured Performance of Tilting Pads by Means of Interferometry. III. The Parallel Surface Bearing. By C. L. Robinson and A. Cameron. Pp.361-395. UK £2.30; Overseas £2.40 (London· The Royal Society, 1975.) [244

New Behaviour, Vol. 1, No. 1, 24 April 1975. Pp.1-48. Published weekly. (London· New Science Publications, 1975.) [243

Ministry of Agriculture, Fisheries and Food. Report on Research and Development 1974. Pp.59. (London· HMSO, 1975.) 65p net. [244

Council for the Protection of Rural England. Annual Report for 1974. Pp.27. (London. Council for the Protection of Rural England, 4 Hobart Place, SW1, 1975.) [254

Broadcasting: The Executive Function. By Sir Charles Curran. (BBC Lunch-Time Lectures, Ninth Series, 6.) Pp.16. (London: BBC, 1975.) [294

All Heaven in a Rage: a Study of Importation of Wild Birds into the United Kingdom. Pp.41. (The Lodge, Sandy, Bedfordshire: Royal Society for the Protection of Birds, 1975.) 85p. [304

Proceedings of the Royal Society of London. A: Mathematical and Physical Sciences, Vol. 343, No. 1633 A Discussion on the Scientific Results from the Prospero and Ariel 4 Satellites. Pp.150-287+5 plates. (London. The Royal Society, 1975.) UK £3.20; Overseas £3.30 [304

Bulletin of the British Museum (Natural History). E ntomology, Vol. 31, No. 9: The Protura of the Bismarck Archipelago and Solomon Islands. By S. L. Tuxen and G. Imadaté. Pp.331-375. (London: British Museum (Natural History), 1975.) £3. [304

Other countries

United States Department of the Interior: Geological Survey. Professional Paper 751-C. Geohydrology of the Artificial-Recharge Site at Bay Park, Long Island, New York. By John Vecchioli, G. D. Bennett, F. J. Pearson, Jr and L. A. Cerrillo. Pp. iv+29. (Washington, D.C.: Government Printing Office, 1974.) 95 cents. [144

Environment Canada: Fisheries and Marine Service Technical Report No. 518. Chemistry, Applications, Toxicity and Pollution Potential of Thallium. By V. Zitko. Pp. 41. (St. Andrews, New Brunswick: Research and Development Directorate Biological Station, 1975.) [144

More Early History of Australian Zoology. By Gilbert P. Whitley. Pp. 92. (Sydney: Royal Zoological Society of New South Wales, 1975.) $2.50. [154

Smithsonian Contributions to Botany. No. 18: The Genus *Aphelandra* (Acanthaceae). By Dieter C. Wasshausen. Pp. v+157. $2.80. Smithsonian Contributions to Zoology. No. 186: A Revision of the South American Fishs of the Genus *Nannostomus* Gunther (Family Lebiasinidae). By Stanley H. Weitzman and J. Stanley Cobb. Pp.iii+36. $1.10 (Washington, DC: Smithsonian Institution Press, 1975. For sale by US Government Printing Office.) [164

Stadley Genetics Symposia, Vol. 6. Edited by G. P. Rédei Pp. 168. (Columbia, Missouri: University of Missouri, Agricultural Experiment Station, 1974.) $4.50 [164

Australia: Commonwealth Scientific and Industrial Research Organization. Annual Report of the Division of Animal Physiology, 1973/1974. Pp. 86. (Sydney: CSIRO, 1975.) [164

Canada· Department of Energy, Mines and Resources. Geological Survey of Canada. Paper 74-7: Geological Survey of Canada Radiocarbon Dates XIV. Pp. 11. $2. Paper 74-55. Geological Interpretation of Bouguer Anomaly and Magnetic Anomaly Maps East of the Magdalen Islands, Southern Gulf of St. Lawrence. By Anthony B. Watts and Rochard T. Haworth. Pp. 9. (Marine Sciences Paper 10.) $3. Geological Map 1374A. Dahadinni River, District of Mackenzie. (Ottawa: Information Canada, 1974.) [174

Australian Academy of Science. Science and Industry Forum. National Goals and Research Needs. (Papers delivered at a Forum meeting 16 February 1974.) Pp. 63. PhD Education in Australia—The Making of Professional Scientists. Pp. 211. (Canberra: Australian Academy of Science, 1974.) [184

Microbia. Tome 1, No. 1, 1974. Pp. 1-36. (Nancy

Cedex. Association des Diplomes de Microbiologie de la Faculte de Pharmacie de Nancy, 1975.) [214

Bulletin of the Fisheries Research Board of Canada, No. 192: Catalogue and Synopsis of *Caligus,* a Genus of Copepoda (Crustacea) Parasitic on Fishes. By L. Margolis, Z. Kabata and R. R. Parker. Pp. 117. (Ottawa: Information Canada, 1974.) $5. [214

Person to Person

Edinburgh–Amsterdam. Apartment house in Edinburgh, available August/September for about a year, suitable for bachelor or couple, centrally heated, phone, small garden, close to University. If necessary, will exchange for same in Amsterdam (phone Edinburgh 031-332 8898, *or* Amsterdam 010-515 3166 *or* 193 223).

American anthropology. Maryland Afro–American and Indian Study Center is initiating extensive genealogical and genetical studies of Blacks and American Indian families. Contributions for research library of relevant publications and reprints greatly appreciated. Also, ecology and sociology of these populations · (K. R. Dronamraju, Research Coordinator, Maryland Commission on Afro–American and Indian History and Culture, 12 West Madison Street, Baltimore, Maryland 21201).

Glasgow accommodation. Wanted October 1, 1975 to March 31, 1976, two bedroom flat or house within reasonable distance of University of Glasgow (Dr J. T. M. Neilson, Associate Professor, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611).

Paris–Cambridge. Accommodation wanted in Paris for Biochemist and wife during FEBS conference, July 20–26, in exchange for fortnight in Cambridge for adult or teenager (Dr I. West, 50 Mulberry Close, Cambridge CB4 2AS, UK; tel. no. 65860).

There will be no charge for this service. Send items (not more than 60 words) to Robert Vickers at the London office. The section will include exchanges of accommodation, personal announcements and scientific queries We reserve the right to decline material submitted. No commercial transactions

---

Records of the Australian Museum, Vol. 29, No. 10 The Earthworm Genus *Oreoscolex* (Oligochaeta: Megascolecidae) in New South Wales. By G. B. M. Jamieson. Pp. 245-260. (Sydney: Australian Museum, 1975) 50c. [214

New Zealand: Department of Health. Fallout from Nuclear Weapons Tests Conducted by France in the South Pacific from June to September, 1974, and Comparisons with Previous Test Series. Pp. 28. (Christchurch· National Radiation Laboratory, 1974.) [214

Connecticut Agricultural Experiment Station. Bulletins. No. 734: Pesticides—Inspection Report Pp. 14. No. 735: Aerial Application of *Bacillus thuringiensis* Against Larvae of the Elm Spanworm and Gypsy Moth and Effects on Parasitoids of the Gypsy Moth. By D. M. Dunbar, H. K. Kaya, C. C. Doane, J. F. Anderson and R. M. Weseloh. Pp. 23. No 736: Commercial Feeding

Stuffs—Inspection Report, 1972 By J Gotdon Hanna. Pp. 8, No 737. 77th Report on Food from Connecticut Markets and Farms, 1972. By J. Gordon Hanna. Pp. 180. No. 738. Commercial Fertilizers—Inspection Report, 1972. By J. Gordon Hanna. Pp. 13. No. 739: Comparative Efficiency of Energy Use in Crop Production. By G. H. Heichel. Pp. 26. No. 740· Cockroach Proofing—Preventive Treatments for Control of Cockroaches in Urban Housing and Food Service Carts. By R. C. Moore. Pp. 13. No. 741: Pesticides—Inspection Report, 1973. By J. Gordon Hanna. Pp. 17. No 742 Commercial Fertilizers—Inspection Report, 1973. By J. Gordon Hanna. Pp. 15. No. 743· Commercial Feeding Stuffs—Inspection Report, 1973. By J. Gordon Hanna. Pp. 8. No. 744: Gypsy Moth—Aerial Tests with *Bacillus thuringiensis* and Pyrethroids. By H. Kaya, *et al.* Pp. 22. No. 745: 78th Report on Food from Connecticut Markets and Farms, 19731 By J. Gordon Hanna. Pp.218. (New Haven, Conn.: Connecticut Agricultural Experiment Station, 1973 and 1974.) [224

Smithsonian Contributions to Zoology, No. 183: Index of the Genera and Species of the Freshwater Triclads (Turbellaria) of the World. By Roman Kenn. Pp. 90. (Washington, DC: Smithsonian Institution Press, 1974. For sale by US Government Printing Office.) $2.05. [224

Studies of Thermodynamics. By Antonio Giacalone. Pp. 107. (Palermo: I.R.E.S., 1975.) [224

Gmelin Institute for Inorganic Chemistry of the Max Planck Society for Advancement of Science, Frankfurt (Main)—Descriptive Brochure. (Frankfurt/Main: Gmelin Institute, Varrentrappstrasse 40/42, Postfach 90 0467, 1974.) [224

Das Fluglarmprojekt der Deutschen Forschungsgemeinschaft. Eine Interdisziplinare Untersuchung uber die Ausworkungen des Fluglarms auf den Menschen. Von August Wilhelm von Eiff, *et al.* Kurzbericht von Bernd Rohrmann. Pp 62. (Boppard: Harald/Boldt Verlag, KG, 1974) [224

US Department of Commerce, Social and Economic Statistics Administration. International Research Document No. 1: Population and Economic Planning—a Macro-Analysis. Pp. 6. (Washington, DC US Department of Commerce, Social and Economic Statistics Administration, Bureau of Censis, 1975.) [224

Sveriges Geologiska Undersokning. Avhandlingar och Uppsatser. Ser. Ca, Nr. 48: The National Groundwater Network of Sweden. By Lars Nordberg and Gosta Persson. Pp. 160. (Stockholm: Sveriges Geologiska Undersokning, 1974.) [224

Annals of the South African Museum. Vol. 66 Part 11: A Revision of Massy's Checklists of 'South African' Cephalopoda. By Martina A. Roeleveld. Pp 233-255. R.2.30. Vol. 66 Part 12· Additions to the South African Species of Phthirilinae and Usiinae (Diptera: Bombyliidae) with Keys to All the Known Species. By A. J. Hesse. Pp. 257-308. R.3.70. Vol. 67, Part 1: Tow New Species of Campanularian Hydroids from South Africa. By C. Gow and N. A. H. Millard. Pp. 1-6. R.160. (Cape Town. South African Museum 1975.) [234

Museum of Applied Arts and Sciences, Sydney. Annual Report for 1973. Pp. 28. (Sydney: Museum of Applied Arts and Sciences, 1974.) [234

Canada· Department of Energy, Mines and Resources. Geological Survey of Canada. Economic Geology Report No. 28· Geology of Canadian Tin Occurrences. By R. Mulligan. Pp. 155 (6 plates). $7. Paper 73-39: The Epworth Group Rocknest Lake Area, District of Mackenzie. Pp. J. A. Fraser. Pp. vi+23. $2. (Ottawa· Information Canada, 1974 and 1975. [244

The Science Museum of Victoria. Report of Activities 1973/1974. Pp. 25. (Melbourne: Science Museum of Victoria, 1975.) [244

The Annual Report of the Agricultural Research Council of Malawi, 1972. Pp. 76. (Thondwe, Malawi· Agricultural Research Council of Malawi, 1975.) [244

National Research Council of Canada. NRC Associate Committee on Scientific Criteria for Environmental Quality. Report No. 11 Endosulfan—Its Effects on Environmental Quality. Pp. 100. (Ottawa· Publications, NRC, 1975.) $1.25. [244

Smithsonian Contributions to Botany. No. 19· The Genus *Thrinax* (Palmae. Coryphoideae). By Robert W. Read. Pp. iii+98 $2.40. No. 20: Flora of Micronesia, 1: Gymnosprmae. By F. Raymond Fosberg and Marue-Helene Sachet. Pp. iii+15. 70 cents. (Washington, DC: Smithsonian Institution Press, 1975. For sale by US Government Printing Office.) [254

Pain, Vol. 1, No. 1, March 1975. (The Journal of the International Association for the Study of Pain) Pp. 1-108 Published Quarterly. Subscription rate: Dfl. 90 per year (including postage). (Amsterdam· Elsevier-North-Holland Publishing Company, 1975.) [284

CERN—European Organization for Nuclear Research. CERN 75-2 Power Laws in Particle Physics. By D. Amati and S. Fubini. Pp. vii+31. (Geneva· CERN, 1975.) [284

Hydrological Effects of Urbanization. (Report of the Sub-group on the Effects of Urbanization on the Hydrological Environment of the Co-ordinating Council of the International Hydrological Decade.) Prepared under the chairmanship of M. B. McPherson. Pp 280. (Paris: The Unesco Press; London· HMSO, 1974.) £10 15.

Records of the Queen Victoria Museum, Launceston. No 52. A Catalogue of the Type Material in the Queen Victoria Museum, Launceston. By R. H. Green. Pp. 15. No. 53 Latumcephalum (Boopidae Phthiraptera: Insecta). By Theresa Clay Pp. 2. No. 54. An Account of an Archaeological Reconnaissance of Hunter's Isles, North-West Tasmania, 1973/74. By Sandra Bowlder. Pp. 22. (Launceston: Queen Victoria Museum, 1974)

Australia Department of Defence. Australian Defence Scientific Service. Materials Research Laboratories Report No. 619: Trace Analysis of Heavy Metals in the Marine Environment. By R. W. Pettis and A. T. Phillip. Pp. 33. (Ascot Vale, Victoria: Materials Research Laboratories, 1974.) [294

July 10, 1975

Good for people, bad for science

ONE of the merits of democracy is not simply that it gives people the chance to choose whom they want to govern but also that it gives them the chance to say whom they do not want. In particular the democratic process makes it possible to say to a government— enough, we don't like the way you do things, it's time to try another way. The ability to reject as well as to accept seems to be a necessary part of most human endeavour, at least any endeavour in which there is any concept of growth or evolution. Foolish would be the person prepared to take on any commitment from which there seemed to be no means of escape, if needed, and foolish are those who try to make the means of escape humiliating, expensive or distasteful.

The development of science also shows the rejection mechanism in action; not simply in the process by which the scientific manuscript has to stand the test of peer review, but also in the endless self-criticism which the good scientist applies to his ideas long before they are committed to paper and in the rapid way in which the scientific community works over published material seeking out errors and inconsistencies.

It is strange that, although we recognise the need that scientific ideas should not only have a platform but should also have to face a barrage of rotten eggs, we are unbelievably cautious when it comes to the scientist himself. If the idea doesn't make the grade, it won't last six months; if the scientist doesn't make the grade—well he's got tenure and could be around for another thirty years.

Job security is, of course, an excellent thing in many ways. It reassures those who set out on long and some- times tedious intellectual pathways that they are not going to have to worry about keeping in favour with their employer or about producing quick results simply in order to stay in business. It keeps the universities and the Civil Service away from any form of external threat to their pursuit of knowledge. But at what cost?

Tenure is a virtual guarantee that the system will either agree indefinitely to your doing what you want to do or that it will find you another job if the present one gets too much. This is a very humane and

enlightened practice but it bears little relationship to a world in which the demands on science are changing rapidly; it also takes no notice of the propensity of science to proceed by revolution. Further, although keeping in favour with one's employer is not necessary to being a good scientist, science, even within universities, does call for a certain amount of man- management, and the need for management is bound to increase. Yet if managers lack the ultimate sanction of being able to dispense with those who cannot or will not contribute effectively, management has one hand tied behind its back.

The pressures in recent years have been rather obviously towards increasing job security in universities (the Civil Service is already about as secure as the priesthood) and there is talk now of giving laboratory technicians some sort of tenure. Such pressures ought to be firmly resisted and, if anything, there should be serious discussions about reversing the trend. It would obviously be silly to put the academic or governmental scientist on to a three-months' notice basis, but few could complain of indecent haste if a contract came up for renewal on both sides once every ten years. There might be stronger support for such an idea within the scientific community than is generally realised.

Such a procedure would also permit the at present almost impossible operation of closing down a depart- ment to be carried out relatively bloodlessly within the space of a few years when necessary.

The standard objection to any loosening of the tenure grip is to point to the problems of those who would be turned out after many years of service. The answer to that is that there are already many careers in which there comes a day of reckoning; perhaps the most analogous is the military profession. If the change is intelligently anticipated and financially assisted there is no reason why it should be a humiliating experience at all. Indeed, for all we know, the release every year of a few hundred scientists into a more general environ- ment might encourage industry and commerce to use them imaginatively. After all, not every retired Major runs a sweet shop.   □

IFIAS: the top people's problem posers

The International Federation of Institutes for Advanced Studies aims to mobilise a global intellectual community to examine problems which have not been officially recognised and subjected to political convenience. Wendy Barnaby reports.

THE last few years have seen an upsurge in discussions about the social responsibilities of scientists. From Koestler's "Callgirls" to articles in the daily press, the familiar message has been that science is not an activity which should be broken up into discrete categories and carried on in laboratories isolated from moral issues and awareness of the directions in which societies are developing. Such sentiments have already—unfortunately— become platitudinous. So it is rather a shock to discover a potentially high-powered organisation which exists to put them into action.

The International Federation of Institutes for Advanced Study (IFIAS) is a loosely-structured conglomeration of some twenty research institutes in sixteen countries. It is a unique federation because the institutes cover at least ten very different disciplines and co-operate on scientific projects which they hope will contribute to the solution of some of our global problems.

The federation was set up in 1972 with $90,000 in equal grants from the Nobel, Rockefeller and Swedish State Bank Foundations. It is non-profit making, and non-governmental, with headquarters at Stockholm in three small rooms in the Nobel House, where its secretary, Dr Sam Nilsson (a physicist), also lives. Its structure and methods of working are very flexible: it is not so much an organisation, says Dr Nilsson, as a lack of organisation. General guidance on the course to be followed and on specific projects is given by the directors of the member institutes, a board of trustees (whose Chairmen are Drs Alexander King (UK), Paul M. Fye (USA), Abdus Salam (Pakistan) and Victor Urquidi (Mexico)), and a panel of advisers in different fields who have participated in IFIAS work. The secretariat is the go-between for the institutes, and initiates and catalyses new projects. It has been supported, after the exhaustion of the initial funds, by $150,000 a year, a sum made up of annual fees from member institutes, each of which pays 0 5% per 1,000 units (whether dollars, yen, rupees or whatever) of its operating budget, and grants to be paid for 5 years by foundations and similar bodies in Sweden ($35,000 each year), USA (also $35,000), Japan ($10,000), Italy ($5,000) and France ($5,000). Each institute taking part in a project also pays a levy of 10% of its contribution to the project. These levies are the secretariat's third source of income.

The institutes themselves specialise in fields including oceanography, medicine, physics, humanistic studies, economics, ekistics (the study of human settlements), genetics, international relations, rice research, mathematics and insect physiology and ecology. Together they represent 5,000 researchers and over $100 million in annual expenditures.

IFIAS' intention is to mobilise this global intellectual community to examine problems which have not been officially recognised and subjected to political convenience. It is the belief of the federation that more effective communication must be stimulated between, on the one hand, those who make the decisions involving long-term consequences for mankind, and, on the other, the world's intellectual and spiritual leaders. By choosing as-yet politically-uncontaminated areas of study, and by consulting and involving the decision makers in projects from the very beginning, IFIAS hopes to bring the influence of facts and well-considered alternatives to bear on the decision makers when they have to act. The emphasis is on working with decision makers rather than presenting them with tomes of conclusions which will never be read. By making use of the contacts of its members, the federation has been able to adopt a policy of dealing only with top people, whether politicians, bureaucrats, businessmen or scientists, and so has a greater potential for influence than most organisations.

Specific action requires a specific focus. Every project is therefore concrete, complex, budget- and time-limited, global in scope and involving inter-disciplinary research. Participation in projects is not limited to member institutes. Other research bodies take part when their expertise is needed, and in this way various UN agencies— UNESCO, WHO, the UN Environment Programme (UNEP) and the World Meteorological Association (WMO)— are involved in different projects. No proposal for a new project is accepted before it has been extensively screened at workshops to make sure that nobody else is doing it better, that at least two member institutes from different countries are willing to assume responsibility for it, that a specific scholar is willing to lead it, and that it has social, ethical and humanistic implications and financial support. When a topic is first accepted, IFIAS sets up a programme of activity which may or may not develop into a full-scale project. So far, funding has come from the World Bank, Volkswagen, the Ford and Rockefeller Foundations, UNEP, UNESCO and the Swedish agency for aid to developing countries (SIDA). To promote contacts with those who wield power over hundreds of thousands of lives, and to attract funds, IFIAS has also begun to approach international corporations.

The programmes and projects so far in operation are:
- Impact of climate changes on the character and quality of human life (budget: $300,000)
- Human settlements: understanding their nature and guiding their development for the benefit of man ($325,000)
- Socio-economic and ethical implications of enzyme engineering ($194,000)
- Energy and quality of life ($80,000).

These programmes and projects are being screened:
- Options for regions faced with water shortage
- Interaction of health, nutrition and education on human growth and development
- Loss of productive soil
- Third world: cultural transfer— cultural change
- Independence and interdependence in the face of regional collapses.

The projects are not concerned with research which gives rise to predictions about resources, climate changes or urbanisation for example. As Dr Nilsson points out, there is a wealth of such predictions already. IFIAS sees its task as examining their consequences. The human settlement project starts from the prediction that by the year 2000, 50% of the estimated world population of 6.5 billion will live in cities. The rationale of the project is that, as these cities will be built anyway, they should be planned in order to conserve resources and promote the best possible living conditions for their inhabitants.

The project aims to draw up a classification of human settlements and, through case studies from Sweden, France, Mexico, India, USA, Columbia and Argentina, to establish criteria for understanding their development. It is hoped that the project will arrive at a conceptual framework which could point to practical suggestions for the UN Conference-Exposition on human settlements (HABITAT) to be held in Vancouver next year.

The project on climatic change is

being carried out against the background of predictions that climatic alterations will soon threaten existing systems of food production. In the face of conflicting predictions about the changes and their effects, the project has adopted the general IFIAS method of dealing with a range of predictions varying from the least to most drastic. What is 'drastic' to begin with is then defined by the general criteria for project selection, mentioned above. The researchers are aiming for plans and actions to establish the technical, social and political means to cope with different climatic threats to food.

The energy programme is in two parts. The first is concerned with energy analysis, which is the idea of working out how much energy is embodied in goods or services, expressed as physical units Undertaken in the light of predictions that, because of limited resources and extravagant use, energy may become a severely limiting factor in many sectors of society, energy analysis includes not only the direct energy used for fuel and to run machinery, but also the energy required to make available each of the input materials. For example, in an industrialised country a loaf of bread is the end product of seed which grows through the absorption of energy (partly from natural sources, partly from petroleum made into fertiliser) into grain, which is harvested, transported and processed into flour by machines, then transported again and added to other ingredients (all of which have arrived at this point through similar processes), mixed and baked by more machines into bread (which is possibly wrapped in paper that is the end product of another energy-consuming chain), and finally transported by petroleum-eating lorries to the super-markets.

Because energy analysis is value-free, it can provide information, in addition to the predictions of our all-too-fallible economic theories, for decision making. It appears to be neither costly nor difficult to carry out, as much of the data needed already exist in national statistics, industrial process data and various other sources. The urgent need is for a common set of rules for calculating energy inputs and transformations, and for common terminology, symbols and units of measure. It is these that the IFIAS project is drawing up.

Once the tools are agreed upon, researchers in different countries will be able to calculate the energy inputs into similar products and identify steps where the use of energy could be reduced. Or the analysis could be used to compare the anticipated energy saving of a development—house insula-

---

IFIAS Member Institutes

Aspen Institute for Humanistic Studies, New York.
Athens Center of Ekistics.
Center for Education in International Management, Geneva.
Center for Theoretical Studies, Miami.
El Colegio de Mexico.
Department of Cell Research and Genetics, Karolinska Institute, Stockholm.
The Graduate Institute of International Studies, Geneva
Instituto de Biofisica, Rio de Janeiro.
International Centre for Theoretical Physics, Trieste.
The International Centre of Insect Physiology and Ecology, Nairobi.
The International Rice Research Institute, Manila

The Japan Economic Research Center, Tokyo.
Johnson Research Foundation, Philadelphia.
Mathematics Institute, Coventry.
National Institute for Research Advancement, Tokyo.
Niels Bohr Institute of Physics, Copenhagen.
Institut Pasteur, Paris.
Tata Institute of Fundamental Research, Bombay.
University Corporation for Atmospheric Research, Boulder, Colorado.
The Walter and Eliza Hall Institute of Medical Research, Melbourne.
The Weizmann Institute of Science, Israel.
Woods Hole Oceanographic Institution, Massachusetts

---

tion, for example—with the initial energy investment necessary to create the development. The energy impact of recycled materials could be assessed. And industries and governments would be able to examine the energy implications of their decisions.

The second part of the energy project is entitled "Alternative Choices of Level of Energy Consumption in Different Societies" Although it is usually taken for granted that a linear relationship exists between energy consumption *per capita* and GNP *per capita*, this is actually true only in energy-affluent societies. The project is analysing why a certain country can achieve the same GNP *per capita* as another country but with a much lower consumption of energy The study is aiming at comparative analyses of different socioeconomic trade-offs in different countries (for example Scandinavia, Japan, India, Kenya) at varying levels of energy consumption rates (say 4%, 2% and 0% a year)

The project on enzyme engineering is typical of the scope and, in its relevance to developing countries, of the emphasis of IFIAS work. Enzymes are of course already widely used on a 'use-and-throw-away' basis to support a variety of socially necessary processes. But it was not until techniques were developed for immobilising enzymes on a support material that their catalytic activity could be retained for reuse, and this has reawakened interest in the use of enzymes as catalysts in many different areas. The IFIAS project is led by Professor Carl-Göran Hedén of Stockholm's Karolinska Institute and is being carried out by member institutes in France, Israel, India and the USA, as well as other institutes in the USSR, Sweden, Canada and the UK. UNESCO is also participating. The project is investigating the use of enzyme engineering in four fields: developing

enzyme-catalysed energy transfer devices which could increase the practical applications of fuel cells or use electrical energy to synthesise organic chemicals for industry, nutrition and pharmaceuticals; testing a quick, enzyme-dependent method of detecting tropical diseases such as malaria; developing a technique of giving the booster shot at the same time as an initial injection in immunisation (to solve the problems of finding people in developing countries when their booster is due); and improving biological control agents by means of enzyme attachment to insect pathogens.

Professor Hedén is enthusiastic about the potential of enzyme engineering in other areas as well. At a symposium in Tokyo last September he outlined an idea which entails a better use of resources for accelerated development in the Middle East. Pointing out that the methods of chemical catalysis currently used in the production of methanol need such high pressures and temperatures that only large-scale factories are competitive, he proposed the use of enzyme catalysis in conjunction with natural methane flares, which today burn as waste, to produce methanol in small-scale plants. Such methanol could be used not only for making fodder protein and enzymes, but also as a cheap, high-octane and relatively clean fuel for cars, or as a safer medium than methane for the carriage of hydrogen.

Such "equilibrium technology"— processes and industrial equipment which use renewable energy sources and are geared to a maximum of recycling of matter—is typical of the inventiveness and optimism, some would say idealism, of the IFIAS approach. The attractiveness of Professor Hedén's scheme for the Middle East, for example, does not end with methanol technology. It also emphasises a range

of biological uses for solar energy ranging from waste treatment to fish farming. He envisages Arab countries such as Kuwait, whose national development plan is very conscious of, and aims for, a balance between ecosystems, providing the vision and financial resources for a regional development effort based on an international input of scientific manpower. This would help to make each side in the Middle East conflict dependent on the other for its future.

As none of IFIAS' projects has yet been completed it is too early to talk about their final results. Already, however, there are signs that IFIAS' work is not going unnoticed. The Brussels headquarters of the Common Market has asked IFIAS for information about the energy analysis programme, and has reserved $100,000 for a joint IFIAS/Common Market project on energy. The Market is particularly interested in adopting IFIAS' methodology for working out alternative levels of energy consumption, with a view to using the technique in drawing up a European energy policy. The World Health Organisation is also very interested in an IFIAS project; this time the one on enzyme engineering. WHO's attention is concentrated on that part of the project dealing with the new method IFIAS has developed for diagnosing tropical diseases. The method was tested in East Africa last February and looks promising.

The first evaluation of IFIAS will no doubt be the one planned by the organisation itself, to be conducted in 1977—five years after its inception. This idea is good in principle, but should not be expected to produce any rational calculation of its achievements. One of the problems involved is the lack of any concrete output or of any yardstick to measure it. Barring administrative incompetence, each team of institutes will obviously be able to produce lengthy documents supporting the results of its particular project. By then they will also perhaps be able to point to even more examples of their work being referred to by the decision makers.

The continuation of IFIAS will depend not on the results of 'evalua-tions' of its work but on the enthusiasm of its members. And nothing could be calculated to sustain that enthusiasm more than the sort of successes the energy analysis and enzyme engineering projects have already enjoyed. Convincing decision makers of the need for action may be harder in the project on climate change. The invisibility of the problem makes it an unlikely target for that public concern which, in countries where politicians depend on the people for their positions, can be an effective complement to IFIAS' high-level approaches in making the decision makers act. On the other hand, the problems dealt with in the project on human settlements are symbolised by the ugliness, dirtiness and congestion of modern cities. If handled properly, the project could have an impact on public policy. Unfortunately, the impact will be greatest if IFIAS keeps its activities separate from the UN conference towards which they are aimed; for incisive and intelligent proposals have a way of emerging from the politically-sensitive and grindingly slow UN machine in a very emaciated form. □

SOMEHOW or other, they had cajoled the money and effort to try their strange and fabulously expensive experiment. Many people said that the funds should be spent on something more useful, or not spent at all. But there was a great public curiosity in the idea; perhaps Orson Welles had helped as much as anyone with his radio broadcast of invaders from Mars in 1938 that caused a nationwide panic. Is there life on Mars? We were now ready to settle for a few anaerobic bacteria rather than green monsters with fusiform antennæ.

The Viking biology team had been called together by the team leader, Harold Klein, for the last time before the launch on August 11, 1975.

One year ago, our hope that even one of the three experiments would be ready by launch-time was a slender one. The constraints were severe; everything that is sent to Mars must be sterile. There is no use carrying bacterial spores from Earth to Mars to detect their presence later. It cost about a million dollars to sterilise the parachute, principally because no-one had ever sterilised a parachute. "Hardy bugs" kept showing up, including one whose spores lived for 6 days at 125 °C. The launch date had to be just right for slinging a shot through the heavens to intercept a planet in conjunction. Worst of all, the instruments were unprecedented in design, infinite in complexity, and strictly limited in size and weight. In engineers' terms, they cost a million dollars per pound; a compacted 20-kilogram mass of electronic and chemical gadgetry whose price made crown jewels look like dime-store baubles. One of the experiments is called "labeled release". It will incubate a pinch of Martian soil, moistened with water containing radioactively tagged nutrients (formate, glycine, DL alanine, DL lactate and glycolicic acid)

Viking gets ready

from Thomas H. Jukes

for two weeks at 6°–14 °C, and any emitted gas will be monitored for metabolically-produced radioactivity.

The second is "pyrolytic release", based on the idea that photosynthetic fixation of carbon dioxide, and possibly carbon monoxide, should take place biologically under Martian conditions. The surface sample will be exposed to "artificial sunlight" from a xenon lamp for several days in an atmosphere containing $^{14}CO_2$ and a little ^{14}CO. The soil will then be heated to 600 °C to destroy organic compounds resulting from photosynthesis and the emitted gases will be measured for radioactivity. The experiment is designed also to detect dark fixation of $^{14}CO_2$ and ^{14}CO. This experiment is specifically oriented towards Martian organisms that may differ from terrestrial ones in being intolerant of water or "terrestrial nutrients". The third experiment is named "gas exchange" but is often called "the chicken soup experiment" because it frankly seeks to woo Martian bugs with a rich, non-radioactive meal containing amino acids, vitamins and cofactors, salts and other nutrients, from kitchens on the Earth. The soil sample is moistened with the culture medium, and the atmosphere in the chamber, mostly helium, is periodically analysed by gas chromatography to see if gases such as methane and CO_2 are emitted.

At the meeting on June 17, the engineers from Thompson-Ramo-Wooldridge and Martin-Marietta appropriately handed us a glossary of 80 acronyms to help us follow their talks. The mood was one of relief, and guarded optimism. All three of the experimental instruments, miraculously, had come into working order at the last minute. The Viking Biology package, in duplicate, had been loaded into two spacecraft at the Kennedy Space Center in Florida. All that now remains is that the instruments should survive the launches on August 11 and 21, the eleven-month, 450-million-mile journey, and the "soft" landings through the thin (5 millibars) CO_2 atmosphere of Mars in July 1976.

After that, perhaps, the faintly-whispered signals will tell us what is happening; not only in the biology package but in the colour cameras, the water detector, the infra-red thermal mapper, the gas-chromatograph-mass-spectrometer, the X-ray fluorescence spectrometer, the seismometer and the weather station. But a main worry is: if the signals next year say that life may be present, will people believe the message?

international news

US puts energy plans in several baskets

by Colin Norman, Washington

Seamans: laying the groundwork.

REPORTS, analyses, blueprints and predictions on energy policy have been pouring out of government agencies in the United States at such a rate during the past years or so that they now often raise little more than a flicker of public interest before being confined to the bookshelves to gather dust. But a report published last week by the Energy Research and Development Administration (ERDA) has attracted considerable attention, and rightly so.

The first broad statement on energy policy to emerge from ERDA since the agency was established in January, the report reflects an important change of priority among long range energy programmes. Specifically, ERDA recommends that more emphasis should be placed on solar energy and thermonuclear fusion, and less on the liquid metal fast breeder reactor (LMFBR), for meeting energy needs towards the end of the twentieth century and thereafter.

Moreover, ERDA has recommended that funding for a variety of non-nuclear programmes, such as the production of synthetic petroleum and natural gas from coal and the development of technology for exploiting geothermal energy, should be increased immediately, while the nuclear programme should be revamped to give more support to research and development on such items as reactor safety, safeguards and the disposal of radioactive wastes.

Described by Dr Robert C. Seamans Jr, the Administrator of ERDA as "laying the groundwork" for energy research and development planning, the report also tacitly abandons the Project Independence goal of reducing oil imports to zero by 1985 (a goal which has long been considered unrealistic in any case). Instead, the report suggests that the United States is unlikely to become self-sufficient in energy before about 1995, unless there is a significant, and unexpected, change in the American way of life.

The report begins by noting that the United States now relies most on the least plentiful domestic energy resources to meet its burgeoning energy requirements. Petroleum and natural gas account for nearly 75% of total energy consumption, yet the Department of the Interior and a committee of the National Academy of Sciences recently suggested that domestic oil and gas supplies are likely to dry up in

about 35 years. Consequently, a switch must be made to other energy sources as swiftly as possible, but Seamans pointed out last week that previous transitions to new energy sources—from wood to coal in the nineteenth century and from coal to oil and gas since 1900—have taken about 60 years to complete. It is clear, he said, that "we don't have 60 years to transfer another energy source" this time.

First, however, a word about what the report is not. "What you will find here is not an instant remedy for energy problems", Seamans said, "nor can it be looked at as an exact blueprint" because priorities will inevitably change as research and development uncovers problems and opportunities in exploiting new technologies. Rather, it is an assessment of present priorities in energy research and development, and ERDA will update the report each year.

The report also makes no explicit projection of energy demand; instead, it outlines the steps which seem necessary to meet whatever demand arises. That approach, it should be pointed out, ignores such considerations as whether it would be better to reduce energy demand rather than open up new resources—a consideration which many see as the crucial question in energy policy for the United States. The report simply states that energy policy should "provide for future needs so that future life styles remain a matter of choice and are not limited by the unavailability of energy".

The conventional wisdom in government energy planning has essentially been that increased use of coal will help bridge the gap between supply and demand of energy in the short term, and that nuclear power will gradually take over from oil and gas for electricity generation in the longer term. The ERDA report certainly does not abandon that view of things, but it suggests that a much greater role should be played by such exotic energy resources as geothermal energy and solar power. It offers a set of recommendations to help increase domestic energy supplies in the near term (between now and 1985), the mid-term (1985 to 2000) and the long term (early in the twenty-first century).

● For the near term, the report suggests that highest priority should be given to efforts to expand direct use of coal, to increase the proportion of electricity generated by light water reactors, and to enhance the recovery of oil and gas from new and existing deposits. Equally high priority should be given to energy conservation by increasing the efficiency of transportation, improving the insulation of buildings and extracting energy from waste materials.

Some changes of emphasis are needed in existing research and development programmes to help achieve those goals, ERDA suggests. Most important, the report recommends that some money should be taken out of the budget for the LMFBR programme and used instead to help improve the safety and reliability of light water reactors, and to develop safe methods for disposing of radioactive wastes. Research and development aimed at mitigating the environmental impact of coal burning should also receive more attention, the report suggests.

● For the mid-term, ERDA suggests that highest priority should be given

to the development of technologies for producing synthetic petroleum and natural gas from coal, and extracting oil from shale. President Ford announced in January that the Administration has set a target of producing 1 million barrels of synthetic petroleum by 1985, a goal which ERDA says can be achieved only if joint government–industry arrangements can be worked out to ensure that new technologies are swiftly taken up for commercial production. The report also recommends that research and development on *in situ* methods of extracting gas from coal and oil from shale should be stepped up because such methods would cause less environmental destruction and require less water. Equally high priority should be given to increasing the use of geothermal energy for electricity production, and the use of solar energy for heating and cooling buildings. Those resources "promise a significant impact" for the mid-term and beyond, ERDA suggests, but they are at present under-used and under-funded.

● It is in the assessment of priorities for the long term, however, that the report recommends the most significant policy changes. ERDA suggests that the LMFBR programme, the nuclear fusion programme and the develop-

ment of technologies for generating electricity from solar energy should be accorded equal priority.

For several years, the LMFBR programme has enjoyed pride of place as the single most expensive energy research and development effort supported by the federal government. The goal was to have a demonstration reactor in operation by 1980, to introduce the reactor commercially in the late 1980s, and to have about 400 individual plants in operation by the year 2000. But the programme has recently run into considerable trouble. The original estimate that the research and development effort would cost $3.3 thousand million has now risen to $10.7 thousand million, the timetable has slipped, and the programme has met with public concern over the adequacy of safeguards to guard against the theft of plutonium, and fears about the toxicity of plutonium. Because of such problems, ERDA believes that it would be foolish for the United States to place all its eggs in the breeder basket, and it is recommending that solar technologies and fusion be accorded equally high priority.

But that does not mean that ERDA has abandoned the breeder project. Last week, in a seperate statement, Seamans announced that ERDA will

publish an updated environmental impact statement on the LMFBR programme in about three months' time. The objective, Seamans said, will be to press ahead as rapidly as possible with the research and development effort. Once that phase is completed, the government will be in a better position to assess the environmental and safety aspects of the LMFBR before giving the green light to commercial production of electricity from the reactor, Seamans said. "There is no presently available or prudent alternative to this course of action," he believes.

Nevertheless, because of a number of factors, ERDA has decided that construction of the first LMFBR demonstration plant, to be built near Oak Ridge in Tennessee, will not begin until late next year, which will put it another year behind schedule. Consequently, Seamans has suggested that Congress should reduce the LMFBR budget for this year by $60 million and transfer some of those funds to other parts of the nuclear programme.

In order to carry out the recommendations outlined in its report, ERDA has suggested that Congress should increase its budget for various non-nuclear programmes by $143 million for this fiscal year (which began on July 1). The budgets for fusion and solar energy should be increased by $38 million and $19 million respectively, ERDA suggests, while research and development on fossil fuels, geothermal energy, conservation and such advanced energy systems as magnetohydrodynamics should also receive substantial increases.

The requests will certainly not fall on deaf ears. In fact, Congress will probably be even more generous towards some programmes than ERDA wants it to be. Last month, the House of Representatives passed a budget bill for ERDA which would add $158 million to ERDA's budget request for non-nuclear energy programmes, including an additional $43 million for solar energy. The bill has yet to be passed by the Senate, but similar action is anticipated there.

Finally, Seamans noted during his press conference that often "people say, 'well, we ought to be able to solve this problem. After all, we got a man to the Moon in 10 years' ". but he pointed out that "It is one thing to carry out the development and take a relatively small number of astronauts on highly experimental type missions. It is another thing to put into being in this country an energy delivery system. an energy conservation system that will satisfy the needs of over 200 million people". Seamans was Deputy Director of NASA during much of the Apollo programme. His new job will clearly be more challenging. □

SUPPORT of basic research in the United States will decline by about 8% in 1975, largely because of inflation, according to a report published by the National Science Foundation (NSF). The foundation calculates that government, industry and non-profit institutions will provide between them about $4,100 million for basic research this year, an increase of about 2% over last year's expenditures but an insufficient amount to keep pace with an inflation rate of 10–11%.

Overall, however, the picture does not look too depressing, and it is certainly much better than in many other countries where inflation rates are nearer 20–30%. The NSF calculates that total spending on basic research, applied research and development will climb to about $34.3 thousand million in 1975, which is an increase of 7% (or a decline of 3% in purchasing power). The development end of the research and development spectrum will fare best, reflecting the increasing support of energy programmes and the rapid increase in outlay on the space shuttle. Moreover, for the first time, industry is expected to spend as much on development as the federal government—about $11 thousand million each.

The decline in real support for basic research will reduce the purchasing power of the research budget to about the same as it was in 1969, and it follows several years of essentially no growth. Although the NSF does not break down the budget between fields, it suggests that outlays on military and space research and development will account for a slightly smaller share of the total— about 36%, compared to about 50% in the mid-1960s. Outlays in those two areas are expected to be a mere $12.3 thousand million in 1975.

The analysis is based partly on the Administration's budget request, which was submitted to Congress in February. Although Congress has yet to complete action on any major appropriations bill (even though the fiscal year has already started) the indications are that it will increase the total federal outlays for applied research and development while reducing expenditure on basic research even further. Last month, for example, the House of Representatives deleted some $44 million from the NSF's budget, but increased the applied research budgets of the Energy Research and Development Administration and the National Institutes of Health. The Senate is expected to follow suit.

THE inaugural meeting of the World Food Council was in danger of breaking down in Rome last week after delegates of the developing countries had expressed dissatisfaction with the draft final report prepared by the secretariat. A major row blew up as the secretariat was |accused of uncooperativeness and inefficiency, among suggestions from delegates that the Council was dominated by 'imperialistic and colonial forces.'

"Possibly the best thing that could have happened" is how a senior FAO staff member described the row that brought the WFC meeting into the headlines. Just what sparked it off is not clear. One factor was certainly the frustration of delegates—among whom were ministers from some 30 countries—at the inadequate preparation for the meeting, and the inefficiency and tactlessness with which it was handled. Another was probably the feeling among the Third World countries that the United States was trying to bulldoze the meeting into accepting its view of how to deal with world food. In any event, a meeting which, after a week's argument, finds it necessary to appoint a working party to decide its own rules of procedure, and accepts a declaration charging its president with convening a further session "within a reasonable period" cannot be said to have helped put a single spoonful of rice into the mouth of any one of the world's hungry millions.

In the eyes of many people, there is little need for a another new secretariat to operate a World Food Council, but if it must be set up, it should not draw something like half its top staff from the United States—an unnecessarily tactless way of reminding the hungry countries who they depend on for extra food. One thing that has to be clearly seen is that responsibility for the recent farce in Rome rests not with the impatient delegates of the Third World, nor with their exasperated opposite numbers from the rich countries, nor with the FAO, but squarely on the shoulders of the UN in New York. Coming as it does under UN, the new World Food Council is seen as a political body, and one that, as another senior FAO man put it, "has nothing to do with the old, endemic, stratified, perennial and above all, undramatic, world food problem. You can't go on dealing with a long term problem only when events (such as the Sahel disaster) force the politicians to notice it."

Another important factor in this messy situation is the open and long standing disagreement between the FAO, the agency responsible for world food, and UN headquarters in New

York, which is concerned with the politics and economics of the food problem, but hardly at all, it sometimes seems, with the fate of the hungry millions. At the World Food Conference, also convened by the UN and held in Rome last November, it almost seemed that the FAO was about to be dismembered, with some at least of its functions being taken over by the UN —presumably on the grounds that after 30 years of existence, the agency has still failed to prevent people from being underfed, something it was not set up to do in the first place. It was in Rome that Dr Kissinger, in one of the less fortunate pronouncements of his career, stated that the world food prob-

Facing the facts about food

from Peter Collins, Geneva

lem could be solved in 10 years. What is even more unfortunate is that this was accepted by the UN as a basis for the policy of the proposed World Food Council, to the dismay not only of the FAO but also of the many people inside and outside the UN system who are aware that neither the past performance, nor the current statistics, nor the future promise of the world community justified such a statement.

Here at least the record was set straight by Dr Adekke Boerma, the FAO's Director-General, in his unscheduled speech to the recent Rome meeting. He stated bluntly that the professed aims of the World Food Conference "cannot be brought about within the next 10 years." Nor would he like to say when even the target of 3.6% a year for increased food production could be met. Last week, at the Geneva meeting of the UN's Economic and Social Council, Dr Boerma returned to the attack. Pointing out that "bad weather conditions can hardly be blamed" for the long term lag in agricultural production, he analysed the major constraints both within the developing countries, and in the world community as a whole, which have perpetuated the dismal performance of world agriculture for the past 25 years. Many of these are longstanding, highly intractable problems—lack of fertile land and water resources, absence of suitable technology for the many millions of largely illiterate peasant farmers, land tenure systems which militate against increased production by removing all personal incentives, the whole inadequate rural infrastructure in the developing countries. But much of the blame lies outside these countries. "The attitudes in developed countries

tries which largely control the conditions of international trade, with the exception of oil, provide other constraints. International agricultural adjustment and a more rational world, division of labour are needed to increase employment in developing countries as well as their export earnings," said Dr Boerma.

It was worth noting, he added, not only that the international development assistance had conspicuously failed to meet the targets of the Second Development Decade, but also that less than 10% of official development assistance was allocated to agriculture. Calling for food aid to be "deliberately planned as an integral part of world food policy", he reminded the developing countries that "food aid is a bridging measure that will tide them over until their efforts to increase their agricultural growth rate begin to pay off." And finally, the key was to find and implement the means of increasing the purchasing power of the world's peasantry, for the biggest constraint of all is poverty.

Boerma pointed out that, in spite of prospects for a good crop for 1975-76, world food stock would remain dangerously low, and the "most seriously affected" countries would still face a food gap of between 15 and 20 million tons for 1975–76. What is needed is a vast cooperative effort to bring about "general economic and social development on a massive scale that will enable the legions of the poor ... to earn a decent livelihood so that they can buy the food they need."

Dr Boerma remains unconvinced that the World Food Council, at least as at present planned, can bring about these aims in the short (10 year) term. But while he was speaking in Geneva, the first meeting to try to set up an Agricultural Development Fund was going on in Rome. This may prove to be the one solid achievement of last year's World Food Conference. The debate lies between the Arab, oil-producing countries, who have shown themselves willing to put up their share of the cash, the United States, and the EEC. It is the last of these, notably West Germany, who have been dragging their feet, with Britain in the perhaps unusual role of trying to persuade her partners to spend more. At the recent Rome meeting, it was Britain that set the pace with a pledge of a further $15 million above her already large contribution to the international fertiliser supply scheme. This is an essential part of the effort to help the needy countries produce more of their own food, which, as everyone agrees, is the only way the world food problem can be solved in the long term.

correspondence

Crowther-Hunt's proposals

SIR,—Your leading article (May 29) outlines proposals which Lord Crowther-Hunt anticipates the academic community will eventually endorse. He wants the opinion of university teachers in order to support his own case, but any alteration in the role of universities in Britain will of necessity require discussion among teachers, employers and students.

Lord Crowther-Hunt should ask his colleagues in the government, 'did they suffer or did they benefit from a university education?'

It is facile to quote a figure of 400,000 in full-time tertiary education and the commitment to increase this number to 640,000 by 1981. Has anyone gauged opinions among students, because it would seem that from an economic standpoint there is a distinct disadvantage in pursuing tertiary education. As one graduating student said to me, "I feel cheated because at school I was told that job opportunities would increase enormously if I had a degree and now four years later the choice of jobs is very limited".

In my own department, where some 65–70% of young graduates have obtained a higher degree, we see trained personnel leaving the country because of lack of opportunity. Although this provides some academic benefit to our department, it is almost impossible for these people to return to the UK. I know of two instances where our graduates could only afford to return if they were given professorships, because of the low salary scales in Britain.

In 1967 the Committee of Vice-Chancellors and Principals carried out a survey of the hours worked by university staff. An example of the hours/week was 54 for a vacational period, 63 for term-time and 80 for an examination period. University teachers do not receive remuneration for the vast amount of work which they take home. I wonder how many trade unions would tolerate a situation in which conditions of service are so loosely defined. Recently, Dr Alex Comfort commented on the situation in one research institute where "geriatric cases some of them, drawing fat salaries for years and doing absolutely nothing" created an impression of idleness among all the staff. Such situations are not unknown in universities and could be controlled

with a scheme of voluntary retirement before the official retiring age.

It might be considered relevant to design courses to meet the needs of the country but it would surely follow that relevant employment with a career structure should be available at the end of the course.

In 1968, however, a learned society formed a temporary committee for liaison between employers and teachers. The conflicting demands of employers were such that it was considered to be almost impossible to design a completely acceptable course for all employers. One must realise, therefore, that the demands of the employer should not dominate the discussion.

The increase in student–staff ratios from 8.4 : 1 to 10–11 : 1 is irrelevant when considering increased productivity and the number of staff to be retained in universities. The student–staff ratio or FTE (formal teaching equivalent) does not include the requirement for repetitive teaching to small aliquots of one large class, that is, the staff time involved to complete one course. I would suggest that increased expansion and productivity, if it is desirable, is a function of staff–student ratios, staff-time per student, space to accommodate larger classes, job opportunities, salaries, rationalisation of university courses, the needs of the country and continued investment in universities.

The last proposal to examine the balance between teaching and research in universities is very important. In view of the anticipated increase in time spent on teaching it seems that Lord Crowther-Hunt is proposing a reduction in research programmes. The subsequent effects of such a move would be disastrous. The move would not immediately affect the quality of teaching but would eventually affect the quality of universities. This would severely affect student populations and bright students would flee. There is a school of opinion which believes that the present government equates private education in the secondary field with universities in the tertiary field of education. Before Lord Crowther-Hunt and his colleagues in the Department of Education and Science run the bulldozer over the universities and produce comprehensive tertiary education they should consider the consequences.

D. E. S. STEWART-TULL
Microbiology Department,
University of Glasgow, UK

English and editorial boards

SIR,—Dr M. Charles (May 8) wonders what members of the editorial board of certain journals do, apart from collecting an annual fee and refereeing a paper or two. Speaking as a literary hack whose job it is to edit such papers so as to give them a veneer of literary and scientific competence, I question whether these august editorial names ever referee any of the papers submitted because (a) they would not be able to understand some of the appallingly ill-written stuff, (b) if they could understand it they would realise that much of it is not worth publishing.

Papers submitted in English by authors with another mother tongue are certainly often unintelligible, but it must be confessed that some native efforts from certain of our English universities and technical colleges run them pretty close. This is due to the recent proliferation of such institutes, with the result that anyone who is not actually moronic can now qualify for higher education. The effect is that people who in saner times would be happily and usefully employed behind the counter of the local grocer's now feel impelled to give the world the fruits of their intellectual labours.

J. C. ANDREWES
Cambridge, UK

Not worried

SIR,—I really rubbed my eyes at your contributor John Hall's statement that in Cambridge "The number taking chemistry is so small that professors are starting to worry about the security of their salaries .. " (*Nature*, May 22). The facts are precisely the reverse. The number of third year students opting for chemistry at Cambridge has risen steadily over the past three years and there is already a firm indication of 70 for next year's numbers (the final figure is usually above this initial assessment). The postgraduate situation here shows a similar robust health, particularly in organic chemistry, where it is clear from the shoal of applications that every available place will be filled. I can therefore reassure John Hall that he need not rattle a collecting box for Cambridge chemistry professors just yet.

R. A. RAPHAEL
University Chemistry Laboratory,
Cambridge, UK

news and views

ALMOST six years have passed since the first manned landing and the return of lunar soils and rocks to the Earth. Six manned American and two automated Soviet samplings have amassed some hundreds of kilograms of lunar samples, many thousands of pictures and vast collections of instrumental data The rocks and soils have been photographed, microphotographed, measured for almost every conceivable physical property, picked over, separated, melted, dissolved, and analysed for elements and minerals. What do we now know about the Moon after this first detailed look at another body in the Solar System, using combined space technology and laboratory analyses?

The meeting at the Royal Society attempted to answer this question, but inevitably it has raised many others.

History of the Moon
One major theme was the lunar time scale. An initial Moon-wide melting around 4.6 Gyr to 4.4 Gyr ago, to an undetermined depth, seems to have led to extensive chemical differentiation, producing the aluminium-rich lunar highlands underlain by a mantle rich in iron and magnesium An intense asteroidal and meteorite bombardment then produced the dense cratering of the highlands and ended rather abruptly at 3.9 Gyr ago, possibly during a final burst of increased activity following some undetermined 'cataclysmic' event. The next phase from 3.9 Gyr to 3 2 Gyr consisted of partial melting of the lunar mantle at depth, producing volcanic lavas which spread out as a thin sheet over the rubble, filling the major impact basins. During the last 3 2 Gyr the lunar lithosphere has been too thick and impenetrable to permit these extrusions to continue in all but a minor way Events in this period have been mainly restricted to occasional large impacts, producing craters such as Copernicus and Tycho, and a steady infall of minor amounts of meteoritic debris.

Although this broad outline is widely accepted a number of fundamental difficulties remain. J. Wasserburg of Caltech raised a number of questions in connection with the early differentiations and the later generation of mare basalts. Was the initial differentiation largely complete by 4.4 Gyr ago or did it extend in a continuous way down to the time of the mare basalt flows? The isotopic data

Probing the Moon

from Correspondents

The Royal Society held a discussion meeting on 9–12 June entitled "The Moon—A New Appraisal from Space Missions and Laboratory Analyses"

appear to favour the former view. Refined uranium–lead data indicate that the iron-rich mantle, from which the mare basalts were later generated, was formed 4.4 Gyr ago. This being the case, where in the Moon are the lead daughter products which were generated from 4.6 to 4.4 Gyr? The mechanism for remelting the mantle at later times to produce the mare basalts is also a major unsolved problem, and what is the significance of the apparent absence of mare basalt clasts from the highland breccias? J. Geiss (University of Berne) stressed the mounting evidence from Apollo 11 samples that at least for this site chemically distinct basalt extrusions occurred over a period of 300 Myr. The crater counts in this area of the Moon by G. Neukum (Max-Planck Institute, Heidelberg) support this view

A current controversy over the precise significance of the $^{40}Ar-^{39}Ar$ ages of highland breccias was aired by several speakers G. Turner (University of Sheffield) interpreted the ages of the Massif boulders from Apollo 17 as indicating the time of the Serenitatis basin impact, a significant marker in the lunar stratigraphic sequence. This view was supported by trace element arguments by E. Anders (University of Chicago). In contrast, O. Schaeffer (State University of New York, Stony Brook) stressed arguments supporting a significantly older age of 4.28 Gyr for Serenitatis, and P. Horn (Max-Planck Institute, Heidelberg) emphasised the importance of smaller events in resetting the argon clock. The various interpretations of the highland ages carry important implications for the crater counts made by and discussed by G Neukum An old age for Serenitatis would probably be consistent with a steadily decreasing cratering rate for the early highland bombardment whereas the younger age

seems to require a late stage cataclysm, or dramatically increased cratering rate, at 4.0 Gyr.

Lunar petrology
The basalt lavas that flooded the mare basins from about 3.9 to 3.2 Gyr ago show evidence for a genetic relationship with both the feldspathic crust and the ultramafic mantle. Petrologists and geochemists spoke for and against the Taylor–Jakes model of two-stage melting to explain these relationships. G. M. Brown (University of Durham) showed how the model could explain the 4.6 Gyr model ages of the basalts, the europium anomalies, and the contrasted crystallisation ages and generation depths of the high- and low-titanium basalts, if a terrestrial-type cumulate model involving convection is examined. Some deficiences in the model were exposed by S. Kessen (State University of New York, Stony Brook) who indicated the need for a titanium-rich magma just below the crust (65 km) and the role of basalt contamination by crustal rocks. A. E. Ringwood (University of Canberra) doubted that the Moon was totally melted deeper than about 200 km early in its history (as required by the two-stage melting model), and argued for a lunar mantle similar in composition and density to the Earth's. Also that existing models for the total Moon composition were too rich in CaO and Al_2O_3 to give mare basalts by partial melting M. J. O'Hara (University of Edinburgh) referred to his experimental evidence in support of two revisions of lunar basalt origins. First, crystal sorting within the surface lava flows precludes the use of hand-specimens to predict the nature of the lunar interior Second, the existence of highly feldspathic liquids is evidence for the original presence of water in lunar highland magmas. These discussions and the restraints imposed by the lithospheric rigidity history and crustal-thickness variation, from gravity determinations by S. J. Sjögren (California Institute of Technology), indicated that the whole question of the Moon's thermal history and magma generation is still an open one

Lunar magnetism
The study of magnetic properties of lunar samples now seems to be geared to the most important goal of estimating the intensity of the ancient mag-

netic field. D. W. Collinson, A. Stephenson and S. K. Runcorn (University of Newcastle) presented data suggesting a trend of decreasing field, from 1.3 Gauss at 4 Gyr to about 0.05 Gauss at 3.2 Gyr, but there was little sign of any concurrence with this idea from the other speakers. M. Fuller (University of California, Santa Barbara) showed palaeointensity results in which there was no discernible trend although these were based on an approximate method. The interpretation of lunar magnetic anomalies is also at a very interesting stage; D. Strangway (University of Toronto) showed that some anomalies can be accounted for by a layer of horizontally magnetised material in the floor of some craters. On a more global scale P. J. Coleman (University of California, Los Angeles) showed that satellite observations give the upper limit to the lunar dipole moment as about 10^{19} Gauss cm³. This low value virtually rules out the original magnetisation of surface rocks in a uniform external field as shown by Runcorn and indicates an ancient field of internal origin—a very important contraint on the lunar history models.

Microscale processes

Micrometeorite impact is responsible for the observed particle size distribution within the soil and the formation of secondary particles (glassy welded aggregates), and may be the major mechanism of lateral transport of dust grains. But there was controversy as to whether micrometeorite particles travelling normally to the plane of the ecliptic have the same size distribution as particles in the plane of the ecliptic and as to whether the micrometeorite flux has varied with time. No evidence has been found for the deposition of impact-produced material although in μm-size craters up to 50% of the material from the projectile is retained

The major erosional effect at the Angstrom level is due to sputtering by the solar wind and estimates of the rate of mass wastage generally are in good agreement. The chemical changes which can be effected by solar proton bombardment were discussed at length by C. T. Pillinger (University of Bristol), R. M. Housley (North American Rockwell) and T. Gold (Cornell University), particularly the formation of free iron which has implications for carbon chemistry, magnetic properties and the albedo of the soil. Although rare gas systematics of the soils are now fairly well understood there is still no completely adequate explanation of the excess of ⁴⁰Ar found, as pointed out by P. Signer (Swiss Federal Institute, Zurich). Chemical elements such as carbon and nitrogen have been accepted as indicators of

solar wind bombardment. T. Kirsten (Max-Planck Institute, Heidelberg) reported that nitrogen seems to be the more effectively retained element since N/C ratios for the lunar soil are in excess of those measured for the Sun.

The conference closed with a look into the future by Noel Hinners, head of NASA's planetary programme He pointed out that lunar research has proved to be an excellent prototype for exploration of the Solar System. The Moon also serves as a proving ground for the necessary space technology. Further manned expeditions are unlikely before the late 1980s when lunar bases will probably be set up. Meanwhile, around 80% of the returned lunar samples still await detailed analysis. Plans are going ahead for an unmanned lunar polar orbiter which would carry out detailed Moon-wide surveys of a number of important elements and physical phenomena. Several UK scientists are hoping to participate in this project.

Collaborating T and B cells

from Fritz H. Bach

RECOGNITION of antigen by lymphocytes of the immune system is the basis of the exquisite specificity of response characterising that arm of our bodily defence against foreign invaders. With the realisation that lymphocytes can be grossly divided into two classes, T (thymus dependent) and B (bone marrow derived) cells, and that both of these show great specificity in their response patterns, it was assumed by many that T cells must express specificity in a way similar to B cells. Our understanding that B cells carry immunoglobulins on their surfaces which are used as antigen recognition sites, or receptors, led logically to the possibility that T cells use the same set of molecules as receptors. This assumption has, during the last few years, been queried by many; now again there is immense controversy over whether T cells, like B cells, use immunoglobulins as their membrane receptors.

A possible clue relating to the genetic control of T-cell receptors came from the finding that genes of the major histocompatibility complex (MHC) control immune responsiveness to specific antigens. These immune response, or *Ir*, genes seemed to be expressed in T cells. Later experiments demonstrated that *Ir* genes could also be expressed in B cells.

With the appreciation of the distinction between T and B cells came evidence that these two cell types cooperate in antibody production by B cells—a phenomenon referred to as

T–B cell collaboration. For what is known as a T-dependent antigen, both T and B cells must recognise the antigen; the B-cell response (antibody production) is dependent on help from the T cell The first suggestion that this T-cell help for B cells may come by way of a humoral factor presumably secreted by T cells came from work of Dutton and coworkers.

These two apparently separate lines of investigation involving T-cell factor(s) in T–B collaboration and the genetic control of the immune response were brought together by the observation in several laboratories that the T-cell product which facilitated B-cell response carried antigenic specificities determined by MHC genes; further that these genes mapped genetically with the *Ir* genes. The situation is best analysed in mouse. The *I* region of *H-2*, the MHC in mouse, includes the *Ir* loci; in addition antisera have been raised against *I* region products defining Ia (*I* region associated) antigens. It is with the anti-Ia antisera that the T cell product reacts. Since in the experimental systems of at least some laboratories the T-cell factor has antigenic specificity, it becomes a prime candidate for the T cell receptor. That the factor is not immunoglobulin is supported by several lines of investigation detailed by Munro and Taussig. (this issue of *Nature,* page 103).

The perplexing finding that *Ir* gene control is sometimes expressed in T cells and other times in B cells now seems to have a logical explanation. A number of groups (for references see the article by Munro and Taussig) now have evidence that two separate *Ir* genes linked to *H-2* are involved in the control of a given immune response. One of the two genes controls the ability of the T cell to produce the factor; the other appears to control a 'receptor' on the B cell which combines with the secreted T cell factor. Whereas the T cell factor clearly carries Ia antigenic determinant(s), the receptor on the B cell has so far only been shown to be associated with antigens defined by sera against both the Ia antigens and other *H-2* determinants although Munro and Taussig reasonably argue that the active moieties in these sera may also be anti-Ia.

The evidence provided by these authors and others is elegant in the synthesis they provide for the dual genetic control, the elucidation of the dichotomy between control of factor production versus a defect in factor acceptance and in the implications of these findings for cell collaboration Although at least to some their model may seem awkward in certain minor details, especially in their attempt to explain the specificity of response

defect by limiting the responsiveness toward any given antigen to only some 'classes' of T cell receptors, it stimulates not only further experimental protocols in T–B collaboration but in several other systems. The importance of the Munro–Taussig work, with its demonstration of antigen specificity of the T-cell factor, the individual *Ir* control of T cell factor and B cell receptor for that factor, thus lies not only in the advance this provides for cellular immunology but also in the broader applicability of the model for cell cooperation which can now be studied at the molecular level.

In addition the two MHC *Ir* gene control of immune responsiveness is exciting to the geneticist. The MHC has often been referred to as a supergene as described by Fisher, that is, a group of linked genes that govern biologically interactive processes. During the past few years we have seen the elucidation of at least one such interacting system within the MHC where different antigens controlled by separate MHC genes (the SD and LD determinants) differentially activate

two separate populations of interacting T cells (T–T collaboration); an interaction which leads to the effective development of cytotoxic T lymphocytes. Munro and Taussig in fact speculate that their model for T–B collaboration may apply to T–T interactions. It may be that the antigens to which the separate T cell populations respond can also function as receptors (which may really be their major role) and that an even closer parallel than that envisioned by some will emerge between the LD–SD interaction and the T–B collaborating system.

Thus whereas many questions of key import remain (such as the possible participation of the variable part of the immunoglobulin molecule as a part of the T cell receptor, the question whether there are classes of T cell receptors which explain the specificity restrictions, and many others) the results and models generated by this group and others working in the same area are enormously exciting and provide a focus for the bringing together of several diverse experimental systems related to MHC genes.

Protein folding pathfinders

from Barry Robson

TRYING to discover how a globular protein folds up into its unique, biologically active conformation is not a task for the faint-hearted. The conformation of a protein is determined by hundreds of rotatable bonds, and hence its conformational energy can be described mathematically as a continuous surface with hundreds of dimensions. Through the hills and valleys of this conformational energy surface the molecule must weave its way in search of that single valley which represents the stable, biologically active conformation. A number of recent publications, some relating to experimental observations on folding proteins, and some to computer simulations of the folding process, give considerable insight into the most challenging and contentious aspects of the problem.

Pancreatic trypsin inhibitor is a relatively small protein of known three-dimensional structure and continues to be a popular model system. Creighton (*J. molec. Biol.*, **95**, 167; 1975) continues his experimental investigation of the folding of this molecule, again forcing the formation of disulphide bridges at selected intervals of time in order to covalently cross-link the backbone chain of the molecule and so trap intermediate conformational species. In this paper he directs his attention to the analysis of those intermediate species which have two such links, and observes that the various elements of the biologically active conformation must be assembled in a definite sequence. His findings therefore support the increasingly held belief that there is a unique and obligatory pathway for folding, and that the biologically active conformation is marked not so much by its own stability as the stability of the pathway leading to it.

Creighton's emphasis on a specific folding pathway seems at first glance inconsistent with his additional discovery that the protein in the early stages of folding has considerable conformational freedom in which many different conformations are built up. However, the initial population of the disordered, fully unfolded state contains an enormous variety of conformations which must be funnelled together into the first obligatory step at the start of the pathway proper. The initial intermediates therefore resemble the routes taken by the participants on their way to the official meeting place at the start of a sponsored walk.

Events at the beginning of the folding pathway have also been recently examined in ribonuclease A. Garel and Baldwin (*J. molec. Biol.*, **94**, 611; 1975)

New view of magnetic changes

from Peter J. Smith

THE magnetic field at the Earth's surface generally deviates from the north–south direction (declination), certain components of the field drift slowly westwards (secular variation), and from time to time the main field changes its orientation by 180° (field reversal). But although these phenomena are observable, directly or indirectly, their causes remain obscure. Declination, for example, is generally explained in terms of non-dipole components; but insofar as the origins of the non-dipole fields are uncertain, this merely shifts the obscurity one stage further along. And although it is usual to invoke eddy currents near the edge of the core, field perturbations which trigger greater changes in a homogeneous dynamo, and so on, such 'explanations' are generally intractable to rigorous analysis.

In sharp contrast, Steenbeck and Helmis (*Geophys. J*, **41**, 237; 1975) have now put forward—tentatively—a quite different hypothesis which is not only amenable to mathematical treatment but is also able to link declination, secular variation and field reversal within a common theoretical development. The theory relates strictly to a simple model of a particular form of gyroscope—a rigid body free to rotate within a rotating hollow sphere and weakly

coupled to the sphere by isotropic friction. Steenbeck and Helmis propose, however, that the analysis may also be applicable to the Earth, with the solid core representing the rigid body of the theory.

In Earth terms, the theory predicts that varying irregularities in the magnetic field are a necessary consequence of a slow rotation of the solid core (gyroscope)—a rotation which is both possible and likely. The interaction between field and core arises basically because, being electrically conducting, the core should be penetrated by a part of the field which it then carries along. The axis of the core's rotation will lie in the Earth's equatorial plane and will rotate within it, thereby giving a westward drift of varying velocity (although eastward drift could also arise under certain circumstances). But the rotational state producing the drift ultimately becomes unstable; and the solid core then tips over relatively rapidly, possibly initiating a total field reversal.

There are several problems, chief of which is that mass movements in the fluid outer core will lead to friction which is neither weak nor isotropic. But although this may alter the quantitative argument, it should not invalidate the principle.

have shown that the thermally unfolded state of this protein is an equilibrium mixture of at least two species, one which initiates rapid folding and one which initiates slow folding. In an extension of this study (*J. molec. Biol.*, **94**, 621; 1975) the authors confirm and explore a physical difference between these species. Kinetic analysis shows that the fast-folding species is an intermediate on the folding pathway from the slow-folding species, but, unlike most intermediates found in proteins so far, it represents a considerable fraction of the initial unfolded state. Although the initial unfolded state prepared by heat denaturation of the native protein may not resemble an initial state prepared in other ways, the study does highlight the possibility that the unfolded state itself may not be one of homogeneous disorder.

In conjunction with Creighton's view this would suggest a picture in which our sponsored walkers converge on the official starting point not from a wide variety of different home addresses, but from a limited number of densely populated areas.

The relatively small size of pancreatic trypsin inhibitor makes it an equally popular system with those who would simulate protein folding by computer. After all, proteins contain thousands of atoms, while there are very much smaller molecules at which a theoretical chemist would sensibly balk. Even so, Burgess and Scheraga (*Proc. natn. Acad Sci. U.S.A.*, **72**, 1221; 1975) have produced what at first reading appears an uncharacteristically melancholy document demonstrating that by existing algorithms it is impossible to simulate the folding of trypsin inhibitor.

Their conclusion might appear to contrast with that of Levitt and Warshel (*Nature*, **253**, 694; 1975); but it would not be entirely justifiable to compare these conclusions since they relate to two fundamentally different methods with different immediate aims. Whereas the Levitt–Warshel approach is to reduce the complexity of the energy surface by simplifying the representation of a protein molecule, Burgess and Scheraga favour retaining a more realistic representation of a protein while reducing the computing time by other methods. In particular, they favour starting the simulated folding process from conformations which they hope are already fairly close to the biologically active conformation, at least as far as the conformation of each amino acid residue in the protein backbone is concerned. A variety of algorithms for predicting such starting structures already exists, most of them based on statistical analysis of amino acid residues in proteins of known sequence and conformation. Burgess

and Scheraga find all these predictions wanting as practical starting structures because the ambiguities and inaccuracies currently inherent in the algorithms which produced them must lead to major ambiguities and uncertainties concerning the conformation of the protein as a whole.

The theoretician seeking to fold proteins inside the computer therefore seems to face a dilemma of either using a simplified representation of a protein molecule, and so study folding across a possibly unrealistic energy surface, or of using a more realistic representation, and sending the folding process down the wrong pathway. In practice, no one has proposed using only one of these approaches and it is reasonable to expect that the ultimate practical and repeatable method will utilise elements of both. In any case, current understanding of the folding of proteins suggests that the starting configurations considered by Burgess and Scheraga are more than a convenient artifice. Creighton's experimental work supports the idea that fairly early steps in the folding process are restricted to a limited number of pathways by thermodynamically stable intermediates. If predicted starting conformations can be identified with such intermediates then they represent structures which ought to appear early in any realistic folding simulation. Ultimately successful computer programs using statistical prediction algorithms to select starting conformations would then merely resemble elderly sponsored walkers taking advantage of a handicap system which allows them to join the walk at a later stage without sacrificing any sense of communal achievement.

Fortunately, the prediction of suitable starting conformations is only one of the methods by which computing time can be reduced In particular, Hagler and Robson (*Specialist Periodical Reports*, **6**, 207, The Chemical Society, 1975) have reviewed a class of procedures which, by analogy with methods used for solving complex problems in other fields, may be termed 'heuristic'. But they used the term more specifically for procedures which obtain additional information from outside the conformational energy surface. Up till now, such heuristic procedures have generally been confined to the prediction of starting conformations or the imposition of constraints on the possible final folded structures, but recently Nagano and Hasegawa (*J molec. Biol*, **94**, 257; 1975) have made use of a penalty function which applies throughout the simulated folding. This penalty function includes estimates of the interaction between residues far apart in the protein backbone, but brought close together in three dimensional space by the folding of the protein. These

estimates are dependent on the conformation of the interacting residues and were derived by previous tests on proteins of known sequence and conformation The use of penalty functions is in the best tradition of heuristic programming in its widest sense, and will probably prove very useful. But Nagano and Hasegawa caution that "It would be premature to discuss [whether the penalty function as described] is realistic or useful" Clearly this kind of approach is still in its infancy.

Finding the pathways by which proteins fold taxes the ingenuity and resources of experimentalists and theoreticians alike. In order to communicate their ideas, protein folding pathfinders have developed or borrowed concepts and terminology which frequently confound other life scientists. It would be regrettable if this obscured the prevailing pioneering spirit, for what may at this moment in time be a sponsored walk for a protein molecule is an exploration of uncharted territory for the scientist.

Getting more CO$_2$ into food

from Israel Zelitch

'PHOTORESPIRATION' finally became a familiar word to the top Wall Street executives who read their June issues of *Fortune* or *Business Week* magazine. Articles unexpectedly cropped up there describing the potential importance of slowing photorespiration for increasing food production. At the same time about two hundred scientists (a fairly even mixture of plant physiologists, biochemists, plant breeders, and agronomists) gathered in Madison, Wisconsin to attend the Fifth Steenbock Symposium held from June 9 to 11 at the University of Wisconsin

The subject of the symposium was billed as CO$_2$ metabolism and productivity of plants A number of topics relating to photosynthesis and productivity were talked about, but nine of the twenty-five scheduled talks and most of the questions and discussions in the conference hall seemed to deal with the background and latest work on photorespiration. The greatest controversy was generated by the difference in the views expressed by some leading investigators about the biochemical mechanism for the synthesis of glycolic acid, the chemically simple but enigmatic substrate of photorespiration.

The symposium was organised by R H. Burris (University of Wisconsin), whose laboratory published some of the first plant biochemistry relating to glycolic acid in 1949, and C. C. Black (University of Georgia). Talks by crop physiologists and plant breeders were

interspersed with papers of a more biochemical nature.

The programme covered recent progress in C_3-metabolism (species with fast photorespiration and slow net photosynthesis), C_4-metabolism (species with slow photorespiration and fast net photosynthesis), and crassulacean acid metabolism (a combination of C_4- and C_3-biochemistry that occurs mainly in species normally found in arid habitats).

Ribulose diphosphate carboxylase is the enzyme mainly responsible for fixing CO_2 during photosynthesis, the product of the reaction being two molecules of phosphoglyceric acid. W. L Ogren (University of Illinois) and N. E. Tolbert (Michigan State University) presented the evidence that this enzyme has a dual activity since it also catalyses a reaction between the substrate (ribulose diphosphate) and O_2. The products of the 'oxygenase' reaction are phosphoglycolic acid (a precursor of glycolic acid) and phosphoglyceric acid (the same product formed by the carboxylation reaction). Since O_2 competes with CO_2 for the isolated enzyme in a manner reminiscent of the inhibition of CO_2 assimilation by O_2 in leaves (the Warburg effect), they argue that the 'oxygenase' reaction accounts for all of the glycolic acid produced in leaves and hence all of the photorespiration. Ogren, R. G. Jensen (University of Arizona), and R. C. Chollet (duPont Co.) described biochemical schemes containing carbon balances that they stated could account for glycolic acid synthesis and photorespiration. These schemes assume that all of the glycolic acid comes from the 'oxygenase' reaction and that no more than about 20% of the carbon fixed during photosynthesis is released as photorespiratory CO_2.

Jensen added that he thought there might be other biochemical mechanisms for glycolic acid synthesis. A critical listener noted that none of the schemes account for the well documented observation that photorespiration often consumes at least 50%, and not 20%, of the CO_2 fixed during photosynthesis. Another omission seems to be that the stoichiometry of the products of the 'oxygenase' reaction, on which the above schemes depend, has never been presented. A further weakness of the argument is that the rates of glycolic acid formation by the 'oxygenase' activity in normal air with Jensen's lysed chloroplast preparations are only about 10% as fast as rates occurring in leaves.

There was a strong difference of opinion over whether the ratio of the 'oxygenase' to carboxylase activity is a constant. Tolbert showed that certain metabolites, such as ribose 5-phosphate, could inhibit the 'oxygenase' and not

the carboxylase activity and suggested that metabolites could affect the balance between net CO_2 assimilation and photorespiration. C. C. Black stated that this ratio of activities could vary by as much as 10-fold in leaf extracts from different species. During the course of a special discussion session largely devoted to problems relating to the assay of the 'oxygenase' activity, it became apparent that many factors affect the assay including whether or not the enzyme has had prior contact with bicarbonate.

The C_4 crop species (maize, sugarcane, sorghum) generally have much higher rates of net photosynthesis and productivity than the C_3-species. M. D. Hatch (CSIRO, Canberra) further elaborated the hypothesis that these species are able to raise the CO_2 concentration in their leaf bundle sheath cells by transporting malic acid (or other C_4 compounds) into these cells and then decarboxylating it. The resulting higher CO_2 level in the bundle sheath cells inhibits glycolic acid synthesis and photorespiration and promotes a speedup of CO_2 fixation by enzymes of the pentose phosphate cycle. The CO_2, he believes, does not have much of an opportunity to diffuse out because it is fixed so rapidly.

Painstaking cytogenetic work on the results of several generations of crosses between a C_4- and a C_3-species of *Atriplex* was described by O. Björkman (Carnegie Institution, Palo Alto). The hybrids produced, however, had poorer rates of net photosynthsis than either of the parental types, and he concluded that it is extremely unlikely that superior hybrids will be obtained by conventional interspecific crossing.

Plant breeders who are anxious to incorporate superior photosynthesis into their plants in order to obtain higher yields have had many frustrations and misgivings This was clearly illustrated in the talk by D. H. Wallace (Cornell University) who emphasised that there are numerous systems and interactions concerned with plant productivity besides photosynthesis. He argued that changing only one component of the system, such as photosynthesis, would probably not increase the yield say of dry beans A member of the audience pointed out that plant breeders have not yet had the opportunity to move a characteristic such as superior photosynthesis into different genetic backgrounds in order to make a proper evaluation. A listener also remarked that increasing net photosynthesis by raising the CO_2 concentration in a closed system would surely increase the yield of dry beans, and one must therefore assume that if large increases in net photosynthesis could be obtained in normal air it would be similarly beneficial.

Many participants felt that more opportunities such as this symposium should be created to permit research workers from different disciplines to share information about plant productivity. It appeared to me that the world food problem has finally touched many scientists who might previously have been more aloof from problems existing in the real world.

Assembly of regular viruses

from a Correspondent

The Discussion Meeting arranged by A. Klug and P. J. G. Butler (MRC Molecular Biology Laboratory, Cambridge) for the Royal Society on June 18 and 19 was held to review the more recent work on the spontaneous assembly of viruses, with particular reference to the 'regular' viruses.

As a group viruses have been found to have surprisingly few similarities other than those dictated by size, pathogenicity and the available building materials. Constrained to have their hereditary material as some kind of nucleic acid they may have RNA or DNA (but so far not both) in any of many forms, single stranded, double stranded, circular, in several pieces and so on. Not surprisingly the associated coat structures are equally variable, so that the discussion proved to be more of an analysis than a synthesis.

Although the self-assembly of the tobacco mosaic virus has been recognised for twenty years its mechanism is not yet fully understood, as was made clear by K. E. Richards (Institute de Biologie Moleculaire et Cellulaire, Strasbourg) and P. J. G. Butler and D. E. G. Zimmern (both MRC Laboratory of Molecular Biology, Cambridge). A method for finding the 'recognition site' of the viral RNA is the selective coating of its fragments, a method previously employed by J. G. Atabekov (Moscow State University) who yet again was prevented from attending a meeting in this country. Rather unexpectedly, these fragments have been found by Richards and his colleagues to contain the coding sequences for the coat proteins, which are known from other evidence not to be in the recognition site region.

In the case of one of the small spherical viruses, brome mosaic virus, which is held together by hydrogen bonds between carboxyl groups and by protein–RNA interaction, the coat was shown by B. Jacrot (Institut Max Von Laue-Paul Langevin, Grenoble)

to be assembled from protein subunits existing in a metastable state. He also presented data on the protein–RNA locations in this virus from small angle neutron diffraction in D₂O–H₂O mixtures, a technique with considerable potential. The requirement for metastable intermediates was also put forward by J. King (Massachusetts Institute of Technology) to explain the complex but orderly assembly of the tail of the T4 bacteriophage. Several speakers discussed the details of the band assembly of this and other bacteriophages. An interesting sidelight is the re-use of components necessary for the assembly but not incorporated in the mature virus.

Other speakers dealt with various small viruses including the flexuous plant viruses, one of which, potato X virus, has been reassembled *in vitro*, and spherical viruses, like the turnip yellow mosaic virus, the shell of which is stable in the absence of the nucleic acid, does not rely upon carboxyl hydrogen bonding for its stability and has not yet proved completely amenable to efforts to reconstitute it.

Antigen receptors on T lymphocytes: a solution in sight?

from Melvyn Greaves

A symposium on "Membrane Receptors of Lymphocytes", organised by M. Seligmann, F. Kourilsky and J.-L. Preud'homme, was held in Paris on May 22–24.

THE molecular nature of the cell surface receptor for antigen on T (thymus-derived) lymphocytes has provided one of the central controversies of immunology over the last five or six years. The meeting in Paris produced a major step forward in the resolution of this problem and the closely related question of immune response gene function. Though the meeting included papers on other lymphocyte surface structures including IgD, F₀ receptors, C3 receptors, β₂ microglobulin and Ia antigens, it was the T cell receptor question which once again held the centre of the stage.

Substantial though equivocal evidence had accumulated earlier that the T cell receptor was an immunoglobulin-like molecule—similar if not identical to that on B ('bursa-equivalent' derived) lymphocytes and in serum. The evidence was based partly on the apparent diversity and specificity of T cell responses and also on the capacity of some anti-immunoglobulin sera to block various T-cell activities. But since 1971

three groups of data have considerably influenced research on this question.

First, attempts to chemically identify T cell surface immunoglobulin using lactoperoxidase iodination methods (labelling followed by extraction immunoprecipitation and polyacrylamide gel analysis) have been extensively reported (*Transplantn Rev.*, **14**; 1973). J. J. Marchalonis and colleagues from the Walter and Eliza Hall Institute in Melbourne have maintained that they can reproducibly find considerable quantities of IgM on T cells whereas J Uhr and E. Vitetta (University of Texas, Dallas), R. M. E. Parkhouse and E. Abney (National Institute for Medical Research, London), B. Lisowska-Bernstein and P. Vasalli (University of Geneva) have failed to reproduce this result. Discussion of this discrepancy, centering on technical details, though a regular feature of international conferences, has failed to provide a realistic means of settling the dispute.

Second, the *Ir* ('immune response') gene phenomenon has mushroomed to become a dominant theme of immunology. B. Benacerraf, H. McDevitt and M. Simonson in particular have proclaimed that the T cell receptor was a product of genes linked to the major histocompatibility locus of the species (*H-2* in mice, *HL-A* in man). This speculation was based to a large extent on the view that *Ir* genes were expressed principally at the level of T cells, and on the ability of certain alloantisera directed towards *H-2* or equivalent loci to suppress T cell activity. Third, the discovery that β₂ microglobulin had considerable sequence homology with IgG (see Raff, *Nature*, **254**, 287; 1975) and yet was found associated with HL-A and H-2 on cell surfaces opened up a different philosophical approach to the T cell receptor question. The characteristics of β₂ microglobulin imply that a phylogenetic relationship may exist between immunoglobulin and products of the major histocompatibility locus although there is in man no genetic linkage between genes coding for HL-A, β₂ and immunoglobulin. An evolutionary relationship between HL-A, H-2 and immunoglobulins is further reinforced by recent studies suggesting that the former have an overall tetrameric structure and compact domains that are strikingly similar to those in immunoglobulin molecules (Peterson *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 1612; 1975).

This crucial new information supports something of a compromise solution to the T cell receptor mystery since it could be both Ig-like and H-2 (in the mouse)-like! Several authors have suggested for example that it might have an immunoglobulin-like V

region and a constant region coded for by genes linked to the major histocompatibility locus (see for example Gally and Edelman, *A. Rev. Genet*, **6**, 1; 1972).

Two papers on this problem in particular generated a great deal of interest Hans Wigzell (Uppsala University, Sweden) reported on work with Hans Binz which provided very compelling evidence that the same idiotype or variable region gene product can be found on both B and T lymphocytes. The experimental design they used was based on a series of experiments reported several years ago by H. Ramseier (*Transplantn Rev.*, **10**, 57; 1972); these were, in retrospect, outstanding in the extent to which they were ignored or disbelieved by immunologists. In brief, if (P×Q)F₁ animals (rats in this case) are injected with parental P-type lymphocytes then clones of P cells which are specific for Q antigens will proliferate and secrete antibody (if they are B lymphocytes) or become cytotoxic and release 'lymphokines' (if they are T cells). The recognition in this situation might, on genetic grounds, be thought to be entirely unilateral (only P→Q); but P cells do have something which (P×Q)F₁ cells can recognise and presumably lack themselves. This is the idiotype of the anti-Q antibody and the corresponding receptors on the P anti-Q T and B lymphocytes. (P×Q)F₁ rats can indeed be shown to make antibody to this idiotype(s) either in response to the injection of unseparated P cells (or relatively pure T cells from P rats) or to the infusion of P anti-Q serum antibodies. The antibodies so raised react with P anti-Q antibodies, B cell surfaces (about 1% of B cells) and T cell surfaces (about 7% of cells). All the activity against T cells can be absorbed out by either B lymphocytes or P anti-Q antibodies. Preliminary evidence suggests that the idiotypic determinant on B cells was associated with a molecule the same size as 7S IgG (molecular weight 120,000) whereas the T cell product had a molecular weight around 35,000. The latter was non-reducible and therefore possibly a single polypeptide chain. But it cannot at this stage be ruled out that this is an 'incomplete' receptor which is non-covalently bound to other subunits or chains when *in situ* on the cell surface.

Klaus Eichmann (University of Cologne) presented what appears to be a strikingly similar story. He had raised antibodies in guinea pigs to the idiotype(s) of anti-group A streptococci antibodies produced by cloned (*in vivo*) mouse B cells. Although direct binding to T and B lymphocytes was not tested these antisera had very intriguing effects when injected into mice. Anti-idiotype of the IgG2 class suppressed both T ('helper') and B (antibody-forming cell

precursor) responses; whereas IgG1 antibodies stimulated anti-streptococci B clones (at high concentrations) or both B and T anti-streptococci clones (at low concentrations).

These two sets of observations strongly suggest that T cell clones have on their surface a molecular structure—presumably the antigen receptor—which bears idiotypic antigenicity similar if not identical to that of B cell receptors and B cell-derived serum antibodies. Although unequivocal evidence for the biosynthesis of this determinant by T cells is not yet available these results clearly imply that T and B lymphocytes are equipped with a similar 'immunoglobulin' V gene pool and may have at least some identical genes.

Presumably the amino-terminal end of the T cell receptor polypeptide contains both the idiotypic determinant and the antigen combining site. The crucial question now is the nature of the remainder of the molecule. Is it an Ir region gene product with invariant peptide sequences and domains analogous to those coded by immunoglobulin C (constant) genes? If this is the case and V genes are identical in T and B cells we have the problem of how unlinked V and C (I region) gene products become associated, since in B cells the gene pairs are linked and association is believed to occur at the transcriptional level. Alternatively, could the T cell receptor have an immunoglobulin (of a new class?) C region after all? J. Marchalonis and N. Warner suggested that this latter view was strongly supported by their evidence that almost all T-cell derived neoplasms synthesise immunoglobulins. Significantly, with respect to other discriminating lymphocyte cell surface products (for example θ, Ly antigens) these lymphomas maintain a strict T cell phenotype.

The former view is however probably the popular choice and presumably immunochemical analysis of the Wigzell–Binz idiotypically defined T cell product should soon resolve the issue. This interpretation is also supported indirectly by an elegant series of experiments by M Taussig and A. Munro (see this issue of Nature, page 103). Their important and provocative results suggested that activated T cells released an I region coded glycoprotein (molecular weight about 50,000) which facilitates the B cell antibody response. The 'factor' probably has antigen specificity although this critical point has not been clearly established. Mice of different strains which did not respond to (T,G)–A–L may be deficient in T cells producing the factor, in B cells capable of 'receiving' the factor, or both! An observation of paramount importance was the result of crossing

T cell 'deficient' mice with B cell deficient mice; the F₁ offspring were responders presumably by complementation of T and B cell I region-controlled immunocompetence.

A similar Ir gene complementation effect was reported by B. Benacerraf (Harvard Medical School) and was in fact reported with minimal effect by E. Rude and E. Gunther at the Second International Congress of Immunology last year (*Progress in Immunology II*, vol. 2, 223; Associated Scientific, 1974). The main conclusion emerging from the work of Taussig and Munro is that the Ir region may contain structural genes coding for cell surface glycoproteins (with Ia antigenicity) on both T and B cells. These structures are critically involved in antigen recognition and collaborative interactions between T cells, B cells and possibly macrophages as well. If this is correct then the I region contains genes which not only code for antigen binding sites but also for a 'mutual' recognition system (that is, of T cell Ia molecules by B cell surface Ia molecules).

It seems reasonable to suppose that the molecule studied by Taussig and Munro might be the same as the idiotype-bearing structure identified by Wigzell and Binz, although H. McDevitt (Stanford University) reported that anti-Ia sera could not block (T,G)–A–L antigen binding by T (or B) lymphocytes. Taken together however these results certainly created the strong impression that the T cell receptor mystery may be soon solved. In the meantime, some healthy scepticism is probably called for. T cell populations are clearly very heterogeneous and different types of receptors might exist on subsets programmed for distinct functions. The role of macrophage surface structures in the genetic control of T + B responses merits further consideration. The precise antigen specificity and diversity of putative T cell receptors requires clearer definition as do both their immunochemistry and the cellular site of biosynthesis and uptake.

A hundred years ago

IN connection with the calamitous floods around Toulouse, on the 25th June a singular phenomenon was observed at Clermont-sur-Lanquet. The whole of the earth on the slope of a mountain was moved bodily, a shepherd's house being transported uninjured to a distance.

from *Nature*, **12**, 220; July 15, 1875.

Calcium transport in contraction and secretion

from N. M. Green

The growing interest in the role of Ca²⁺ in a variety of intracellular control mechanisms was reflected in an international symposium on Ca²⁺ transport (Bressanone, May 10–16, 1975) which was devoted to establishing contact between workers in the field of muscular contraction, where the effects of Ca²⁺ are relatively well understood, with those who work on a variety of secretory systems. This report considers only a few of the fifty papers that were presented, emphasising those aspects which may lead to fruitful interactions.

ONE of the main technical problems in the way of a more precise definition of the role of Ca^{2+} in secretory systems is the difficulty of measuring changes in submicromolar, cytoplasmic Ca^{2+} concentrations in the presence of intracellular and extracellular reservoirs where concentrations are increased a thousand-fold. J. Meldolesi (University of Milan) described the analyses of subcellular fractions from pancreas. Interpretation was difficult because of the rapid exchange of Ca^{2+} between compartments after cellular disruption. However, there was an interesting observation of a large non-exchanging reservoir of Ca^{2+} in the zymogen granule fraction. A similar high Ca^{2+} content of secretory granules was observed in pancreatic islet tissue (W. J. Malaisse, University of Brussels) and in parotid gland (Z. Selinger, Hebrew University, Jerusalem). J. L. Borowitz (Purdue University) suggested that uptake of Ca^{2+} by the granules in adrenal medulla might play an auxiliary role in the post-secretory recovery process. A promising new approach to the problem of intracellular Ca^{2+} distribution is that of X-ray microprobe analysis which can detect changes of local concentrations of Ca^{2+} within organelles at a resolution of 50–100 nm, provided that local concentrations, which can be enhanced by microincineration, are high enough (T. A. Hall, Cambridge). A study (with R. Yarom, Hebrew University, Jerusalem) of cardiac muscle revealed unexpectedly high levels in cell nuclei, associated particularly with heterochromatin, suggesting that serious consideration should be given to the nucleus as a reservoir of intracellular Ca^{2+}. M. J. Berridge (University of Cambridge) described another application of the technique to show that

mitochondria of insect (*Calliphora*) salivary gland release their Ca^{2+} following stimulation of secretion by serotonin. Unfortunately, cytoplasmic Ca^{4+} levels are far below the sensitivity of this method, which anyway cannot distinguish free from bound Ca^{2+}. The most suitable technique at present available for measuring free Ca^{2+} is the use of aequorin and it has not yet proved possible to introduce this protein into secretory cells.

Electrophysiological results

Extensive published work by Katz and Miledi has established the existence of time- and potential-dependent channels for Ca^{2+} in synaptic and neuromuscular junctions. The application of similar electrophysiological techniques to secretory systems, partly aimed at testing the wider applicability of these concepts, was described by several groups. E. K. Matthews (University of Cambridge) showed a close correlation between secretion of insulin and spike potentials induced in pancreatic islet cells by D-glucose or by current injection. Since this electrical response depended entirely on the presence of external Ca^{2+} it was possible to calculate from it the magnitude of the Ca^{2+} influx and consequent changes in local internal concentration of the order of 1–2 μM. Glycolytic metabolism was essential to the response implying that the change in permeability was caused by a metabolite rather than by glucose itself. In contrast potential changes in exocrine pancreas induced by acetylcholine or pancreozymin (O. H. Petersen, University of Copenhagen) proved to be Na$^+$ rather than Ca^{2+} dependent implying that the Ca^{2+} known to be required in the activation of secretion was derived from internal stores.

T. J. Rink and P. F. Baker (University of Cambridge) showed that K$^+$ depolarisation of the adrenal medulla gave rise to a time dependent influx of Ca^{2+} which decayed with the half life of 45 s, very much slower than the corresponding effects in nerve and muscle. J. J. Dreifuss and J J. Nordmann (University of Geneva) measured neurohypophysial influx and efflux of ^{45}Ca^{2+} following K$^+$ depolarisation. In this system, as in nerve axons, the Ca^{2+} efflux associated with the recovery phase appears to be driven by Na$^+$ influx rather than being directly coupled to ATP hydrolysis as in muscle. The Ca^{2+} influx was blocked competitively by micromolar concentrations of D.600 (methoxyverapamil). The use of this drug as a Ca^{2+} antagonist follows from the extensive work of A. Fleckenstein (University of Freiburg) who discussed its action on cardiac and on smooth muscle. Its use provided one of the few unifying features of the diverse secretory systems described at

the meeting. Almost all those which required external Ca^{2+} for activation (nerve terminals, neurohypophysis, cardiac muscle, adrenal medulla, endocrine pancreas, insect salivary gland, but not the parotid gland) were inactivated by D.600. It will be of interest to know how far it can be used to characterise molecular species involved in Ca^{2+} conductance. Unfortunately, it has other pharmacological actions as a reserpine analogue. Moreover, N. A. Thorn (University of Copenhagen) reported that in the neurohypophysis it counteracts activation by the Ca^{2+} ionophore A23187, possibly by non-specific complex formation between lipid-soluble amine and carboxylic acid.

The complex interplay between fluxes of Na$^+$ and Ca^{2+} was considered further in several contributions. E. Carafoli (Zurich Institute of Technology) showed that in the absence of permeant anions, Ca^{2+} taken up by isolated heart mitochondria could be released by low (50 mM) Na$^+$. However, Thorn showed that under similar conditions the uptake of Ca^{2+} was also inhibited, throwing some doubt on the interpretation of this result The lack of a defined physiological mechanism for bringing about release of Ca^{2+} from mitochondria makes it difficult to evaluate their role in controlling cytoplasmic concentration, especially since in the long term, as was emphasised by Rink, most of the Ca^{2+} that enters through the plasma membrane must leave by the same route The results of A. B. Borle (University of Pittsburgh) suggesting that cyclic AMP stimulates Ca^{2+} release from isolated mitochondria have not so far proved repeatable. However, Berridge provided evidence for a similar control mechanism to account for his observed release of mitochondrial Ca^{2+} in insect salivary gland Borle emphasised that erroneous conclusions about changes in Ca^{4+} concentrations can easily be drawn from isotopic flux measurements if there is no information about changes in pool size which can be very large.

The actions of cyclic nucleotides in contractile systems are relatively well understood on the biochemical level although the physiological significance is not always clear. S. V. Perry (University of Birmingham) described how the effects of Ca^{2+} in releasing actomyosin from inhibition by the troponin complex are modulated by phosphorylation of the troponin components I and T by either a cyclic AMP dependent protein kinase or by phosphorylase kinase The phosphorylated amino acids have now been located in the sequences of the troponin components, but the way in which phosphorylation and dephosphorylation control sensitivity to Ca^{2+} remains obscure. Cyclic AMP has

a more clearly defined role in controlling the activity of cardiac muscle in response to adrenaline. A. M. Katz (Mount Sinai Hospital) showed that it could enhance the rate of Ca^{2+} uptake by the sarcoplasmic reticulum by activating the phosphorylation of a membrane protein (molecular weight 22,000) by a protein kinase. This phosphorylation was correlated with an enhanced relaxation rate.

It is likely that one direct relation between contraction and secretion will eventually be found at the level of the contractile process itself, since there is a plausible assumption in the field, though little direct evidence, that expulsion of secretory granules is powered by Ca^{2+}-activated actomyosin within the cell. Surprisingly, little was said about this aspect of the relationship, although several interesting papers were given summarising current knowledge in the field of troponin–tropomyosin–actin interactions and the dependence of their association on Ca^{2+} concentration. S. Hitchcock and J. Kendrick-Jones (University of Cambridge) found no functional relation between carp parvalbumins or myosin light chains and the Ca^{2+}-binding troponin component (C) in spite of significant structural homologies, discussed by R. Kretsinger (University of Virginia). Their work on the Ca^{2+}-dependent regulatory properties of myosin light chains in scallop muscle as well as the differences in mode of action of troponins from cardiac and smooth muscle described by S. Ebashi (University of Tokyo), emphasise that control of cellular actomyosin is likely to differ significantly from that of skeletal muscle.

Although initiation of relaxation in skeletal muscle by the sarcoplasmic reticulum is well understood, our knowledge of the mode of control of Ca^{2+} influx in muscle appears little further advanced than it is in secretory systems. The once favoured regenerative release of Ca^{2+} from sarcoplasmic reticulum by local high Ca^{2+} was re-evaluated by M. Endo (University of Tohoku), who showed that unphysiologically high Ca^{2+} concentrations were required and that the process was inhibited by procaine which does not affect the physiological, depolarisation-induced release It would be of interest to know if the Ca^{2+} channels involved resemble those of nerve, heart muscle and secretory tissue in their susceptibility to the action of D.600.

The meeting usefully defined several borderline areas where more work could profitably be done, as well as emphasising the relation of contraction and secretion to more general intracellular control processes involving Ca^{2+} and cyclic nucleotides which were not directly considered.

review article

A hundred years of controversy over sunspots and weather

A. J. Meadows*

After a century of accumulating correlations on the activity of sunspots and atmospheric phenomena around the Earth, we still have no clear idea what exactly the relationships are or what they mean. Much of what is being researched now was already under discussion in the nineteenth century. This article is based on the author's recent Norman Lockyer Lecture at the University of Exeter.

ALTHOUGH the appearance and disappearance of sunspots was one of the first discoveries made with the newly invented telescope in the early seventeenth century, detailed studies with reasonably powerful telescopes had to await the resurgence of interest in solar studies that characterised the latter part of the eighteenth century. One factor in this renewed interest was the possible effect of solar changes on the Earth. William Herschel[1] carried out a detailed study of the solar surface during the period 1795–1800, and, having outlined his results, commented· "Since experience has already convinced us, that our seasons are sometimes very severe, and at other times very mild, it remains only to be considered whether we should ascribe this difference immediately to a more or less copious emission of the solar beams."

He then attempted to find some systematic variation in terrestrial weather that could be linked with changes on the Sun. The only relevant long period data available were some statistics on wheat prices collected by Adam Smith in preparing his book on *The Wealth of Nations*. Herschel assumed these would reflect the average weather conditions in each year, and attributed the fluctuations they showed to solar variation.

This initiative was not followed up for several decades. Herschel's emphasis on the physical properties of celestial objects, although not unique, was hardly characteristic of the first half of the nineteenth century. It was only in the 1860s that the new science of astrophysics became firmly established, and the possibility of a relationship between solar change and terrestrial weather once again came to the fore. By this time, two important observational advances had been made. First, Schwabe in Germany had pointed out that the number of spots visible on the Sun's surface rose and fell in a regular manner with a period of about ten years. Although he announced this result in the early 1840s, it was the early 1850s before it became generally known. Also during the early 1850s, it was found that the frequency of magnetic storms varied with the same period as the sunspots. Thus, by the 1860s, astronomers knew two things that William Herschel did not: that some changes of the solar surface occurred in a cyclical fashion, and that one indisputable relationship existed between solar and terrestrial changes. The latter encouraged the hope that further relationships might be found: the former suggested the way in which they might be sought.

* Department of Astronomy and History of Science, University of Leicester, Leicester LE1 7RH, UK

Lockyer in 1872 asked the readers of *Nature*, "why it is that meteorologists, state endowed or otherwise, have, as a rule, been content to grope their way in the dark, and not only not seek to find, but persistently refuse the clue, which, if followed, would bring them into the light of day[2]" He then explained what this clue was: "Surely in meteorology as in astronomy, the thing to hunt down is a cycle."

The problem from the beginning was that terrestrial weather obviously underwent major fluctuations from day to day. Thus, if cyclical effects extending over several years were to be discovered, it was either necessary to find a clear-cut meteorological phenomenon that showed minimum short-period fluctuations, or to take ordinary meteorological phenomena and look for systematic trends by averaging over large numbers of observations. Several people, Lockyer among them, saw the monsoons in the Indian Ocean as the sort of clear-cut phenomenon that should readily show the effects of solar change Lockyer chanced on this idea : Meldrum, who was in charge of meteorological observations on Mauritius, approached the problem a good deal more systematically. He examined the number, size and duration of cyclones occurring in the Indian Ocean during the period 1847–73, and showed that all these variables were enhanced at solar maximum compared with minimum. He also ingeniously correlated the sunspot cycle with the number of vessels putting into Mauritius for repair after damage caused by heavy seas His extracts[3] from the official port records make livelier reading than most sets of statistics, for example: "December 16–17, 1867 Ship 'Berar', in 24°S and 68°E, experienced a heavy gale, with thunder and lightening; ship hove on her beam ends; cut away top gallant mast, every plate, all clothing, and every book on board washed away. Barometer 29.30 inches."

While Meldrum was putting together figures for the Indian Ocean, Pogson, then Government Astronomer in Madras, was examining weather data for South-east India. His analysis led him to conclude that there was a correlation in that area, too, between variations in the rainfall and the sunspot cycle.

This concentration of effort on the weather of the Indian subcontinent and its environs was not dictated solely by the apparent simplicity of the weather cycle there. The problem of famine was then particularly evident in India— one series of famines in the 1860s being followed by another, even more acute, in the 1870s. As is evident from the meetings of the Indian Famine Commission at the end of the latter decade, the possibility of forecasting future famines

Table 1 Rainfall (Paris) and sunspot cycle for the years 1824–1867

Number of year in mean cycle	Rainfall variation (inch)	Spot variation (number)
1	−2.0	−38 2
2	−0.9	−22.7
3	+0.8	− 5 7
4	+1 9	+33.3
5	+1.9	+41.9
6	+1 8	+30,7
7	+1.1	+13.1
8	+0.2	− 1.5
9	−0.5	−12.1
10	−0.8	−21.7
11	−2.0	−28.0

was under urgent discussion, and the discovery of correlations with the solar cycle seemed to offer the best hope of achieving this.

Studies of weather variation in other parts of the world were being conducted simultaneously, often by the same investigators. Typically, these consisted of an examination either of an extended time sequence of observations made at a single station, or of a shorter time sequence averaged over several stations. Thus Meldrum correlated sunspot numbers with rainfall data measured at the Paris Observatory. As the Observatory had been founded in the seventeenth century, it could provide almost two hundred years of observational material. (The sunspot cycle also had to be extrapolated over this period, of course, and Meldrum, like virtually all his contemporaries, made use of an extensive study of solar records carried out by Wolf at Zurich and published in the 1870s.) Meldrum then studied four spot cycles in more detail, using a shorter time base but averaging over more than 150 observing stations dotted all round the world (see Table 1). In both instances, he found an apparent correlation between the phase of the solar cycle and the weather record.

Rainfall was by no means the only parameter used to characterise terrestrial weather Balfour Stewart used, in addition, atmospheric temperatures and the heights of rivers; whereas W. S. Jevons returned to Herschel's original approach, and linked the number of spots with the business cycle. But the variable that seemed to offer the best chance of providing a physical interpretation of the Sun–Earth interaction was the atmospheric pressure. Blanford[4]—yet another meteorologist working in India—developed the concept of a "barometric seesaw"; by which he meant an oscillation in pressure between two parts of the globe, that varied with the period of the solar cycle

"Between Russia and Western Siberia on the one hand, and the Indo–Malayan region (perhaps including the Chinese region) on the other, there is a reciprocating and cyclical oscillation of atmospheric pressure; such that the pressure is at a maximum in Western Siberia and Russia about the epoch of maximum sunspots, and in the Indo–Malayan area at that of minimum sunspots."

He interpreted this in terms of varying energy supply from the Sun, which led to an alteration in the amount of water evaporating into the atmosphere. This produced, in turn, fluctuations in pressure, temperature and rainfall.

In view of the numerous correlations between the solar cycle and the weather that were discovered in the last quarter of the nineteenth century, it seems at first sight surprising that the century ended with the matter still a focus for controversy. Three reasons can be discerned for the continued doubt.

These correlations represented the result of averaging over widely varying sets of values: their credibility therefore remained dubious in many eyes. As Blanford, who believed in the correlations, remarked, "The amount of the variation is so small in comparison with the irregular

vicissitudes of the weather, that it is for the most part obscured and unrecognisable, except fitfully, or in some few favoured regions."[5] The physical mechanism by which the solar–terrestrial interaction operated still remained unclear in spite of numerous speculations. Moreover, the observations seemed to support contradictory conclusions: the measurements of air temperature suggested greatest solar luminosity at sunspot minimum, whereas the study of rainfall and cyclones suggested greatest luminosity at maximum. It was expected that the discovery of correlations would lead on to the prediction, at least in general terms, of future changes in the weather. Yet even the intensive study of the Indian monsoons failed to lead to an acceptable forecast of famine conditions.

The main sticking point was the uncertainty regarding the exact nature of the supposed solar luminosity variations Not only were the observations contradictory: so were opinions regarding the nature of the variations. The original idea had been that spots blanketed a part of the solar surface, thus reducing the energy emitted, and producing minimum flux at sunspot maximum. By the early twentieth century, it was, on the contrary, even being contended that the luminosity variations might rather be the result of changing numbers of solar prominences, so that maximum luminosity emitted might actually coincide with maximum spot number[6]. The obvious way of resolving this uncertainty was to make a series of direct measurements of the energy received by the Earth during a solar cycle. Throughout the nineteenth century, however, techniques were acknowledged to be insufficiently accurate for this kind of monitoring. There were, in fact, two obstacles in the way of measuring the solar constant. The first was the obvious need to develop a sufficiently sensitive bolometer. But the measurements had to be made at the bottom of the Earth's atmosphere; so, secondly, the effects of fluctuations in atmospheric transmission had to be eliminated. This effectively ruled out the British Isles as a site for the appropriate observations. In fact, interest in possible relationships between solar activity and the weather was clearly on the wane in this country just before World War I, and the renewed attack on the solar luminosity problem took place instead in the USA.

The Smithsonian Astrophysical Observatory in Washington had been involved since its foundation in 1890 in measuring the solar constant. Under Langley, the Observatory's first director, the main concern had been to develop as sensitive and accurate an instrument for this purpose as possible. But C. G. Abbot, the second director, decided not long before World War I that sufficiently good results were being obtained for small changes in the solar constant to be detectable. During the 1920s and 1930s he pushed ahead with the measurements, finding, so he believed, definite evidence for a variation in the solar luminosity from year to year which could be correlated with weather conditions on Earth.

Yet, in spite of this massive effort, *Nature* commented without fear of contradiction at the beginning of World War II that: "Many attempts have been made in the past to correlate solar and terrestrial phenomena . . . There is no direct meteorological effect of any serious importance."[7]

Partly, no doubt, this could be explained by British insularity; for studies of Sun–weather relationships had been at a low ebb in this country since before World War I. But the Smithsonian results were also received with scepticism by many in the USA. The doubts were once more engendered by the difficulty of unravelling a small signal from a large amount of noise: most of Abbot's peers felt that, although his conclusions might ultimately prove to be right, they had not been definitely established. The apparent variations in solar energy might actually be caused by systematic errors, for example in correcting for the influence of the Earth's atmosphere.

Although Abbot continued to stress the significance of the Smithsonian results, the general feeling among both British and American scientists in the years following World War II seems to have hardened towards the view that the total energy emitted by the Sun did not vary appreciably during the solar cycle. This belief, which has strengthened with time[8], obviously rules out the kind of interaction mechanism that was being considered before World War I. It is hardly surprising that interest in the whole subject of solar activity and the weather continued to flag. (Although discussions on the topic continued in the Soviet Union, they were relatively little noticed in the West.) The return of interest only followed, belatedly, after the growth of solar system studies in the 1960s.

The actual correlations studied in the last five years, or so, have not differed very greatly in principle from those we have noted before, although the amount of data that can be collected and reduced has now become a good deal larger Thus one recent attempt to find correlations made use of a study of some ten million different measurements of air temperature[9]. But the crucial point has remained the question of the mechanism involved. However many numbers are invoked, the old doubts are unlikely to be resolved so long as all that can be offered is statistical regularities. If the Sun's luminosity can no longer be regarded as a variable, attention must naturally turn to those features of the Sun that might affect the Earth and that are certainly known to vary during the solar cycle—magnetic fields, particle emission and high energy radiation.

It has long been realised that changes in the upper atmosphere of the Earth can be related to the solar cycle. Balfour Stewart—one of the first to realise that the Earth's upper atmosphere is electrically conducting—attempted to correlate variations in terrestrial temperature and magnetic declination. The interactions here are between the Sun and the ionised upper atmosphere. In the 1960s, it became apparent that the neutral upper atmosphere was also strongly influenced by solar activity, and the belief grew that there might be further interaction with the lower atmosphere. The difference in the masses of the upper and lower atmosphere, however, suggested that such a link could not be direct, rather it would have to occur by some kind of trigger action, so that a small effect in the upper

atmosphere could produce a major effect in the lower atmosphere by way of an instability. An intriguing recent claim—that solar magnetic sector structure can influence terrestrial atmospheric vorticity[10]—may indicate the sort of direction in which this instability should be sought. Unfortunately, there is no scarcity of competitors.

As I have remarked, one thing a satisfactory Sun–weather relationship should be able to do is to forecast future changes in the weather. This obviously requires an ability to predict future solar activity; so it must surely rank as more than a coincidence that interest in such powers of prediction accompanied the upsurge of activity in correlating solar and terrestrial changes both in the nineteenth century and today. Even more interestingly, attempts have been made on both occasions to relate solar activity—measured by sunspot numbers—with particular alignments of the planets[11,12]. Although the exact mechanisms have differed, the significance of such correlations is that, if true, they should permit the forecasting of solar activity for any time in the future, as planetary orbits are very well determined. It is a commentary on the overall problem of establishing anything firmly in this field that, both in the nineteenth century and now, there have been strong counterclaims that the apparent coincidences between planetary positions and sunspot numbers are simply figments of the analysis[13].

Would it then be fair to conclude with Solomon that there is no new thing under the Sun? Not entirely—for, although in one sense additional correlations may add nothing new, they may lead to an acceptable mechanism for the necessary interaction between the Sun and the lower atmosphere of the Earth If this could only be defined, the whole controversial field might be rapidly translated into the realms of high respectability.

1 Herschel, W., *Phil. Trans*, 265 (1801)
2 Lockyer, J. N , *Nature.* 7, 98 (1872).
3 Meldrum, C , *Rep. Br. Ass. Adv. Sci* , 218 (1874)
4 Blanford, H. F , *Nature*, 21, 477 (1880)
5 Blanford, H. F , *Nature*, 43, 583 (1891).
6 Lockyer, J N , and Lockyer, W J. S., *Proc. R. Soc* , 67, 409 (1900)
7 *Nature*, 144, 808 (1939).
8 Smith, E. v. p., and Gottlieb, D M., *Space Sci. Rev* , 16, 771 (1974).
9 King, J. W , *Nature*, 245, 443 (1973)
10 Wilcox, J. M., *et al* , *J Atmos Sci* , 31, 581 (1974)
11 De La Rue, W , Stewart, Balfour, and Loewy, B , *Proc. R. Soc* , 20, 210 (1872).
12 Wood, R M., and Wood, K D , *Nature*, 208, 129 (1965)
13 Okal, E , and Anderson, D. L., *Nature*, 253, 511 (1975)

articles

Packaging of prophage and host DNA by coliphage λ

Nat Sternberg & Robert Weisberg

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, Bethesda, Maryland 20014

Bacteriophage λ can package headfuls of prophage and host DNA. The resulting particles are not infective without removal in vitro of unpackaged DNA that protrudes from the head and prevents the attachment of tails. After tail attachment the particles can transduce a large number of host genes.

THE DNA of the phage λ virion (mature DNA) is a double-stranded molecule about 47,000 base pairs long with specific single-stranded sequences, 12 bases long, at each 5′-phosphate end[1,2] (Fig. 1a). The left end sequence is complementary to the right end sequence; this enables circularisation of a molecule by base-pairing of the ends[3]. Mature DNA is formed by the cutting to size of a longer precursor, a linear polymer of mature molecules joined together head-to-tail
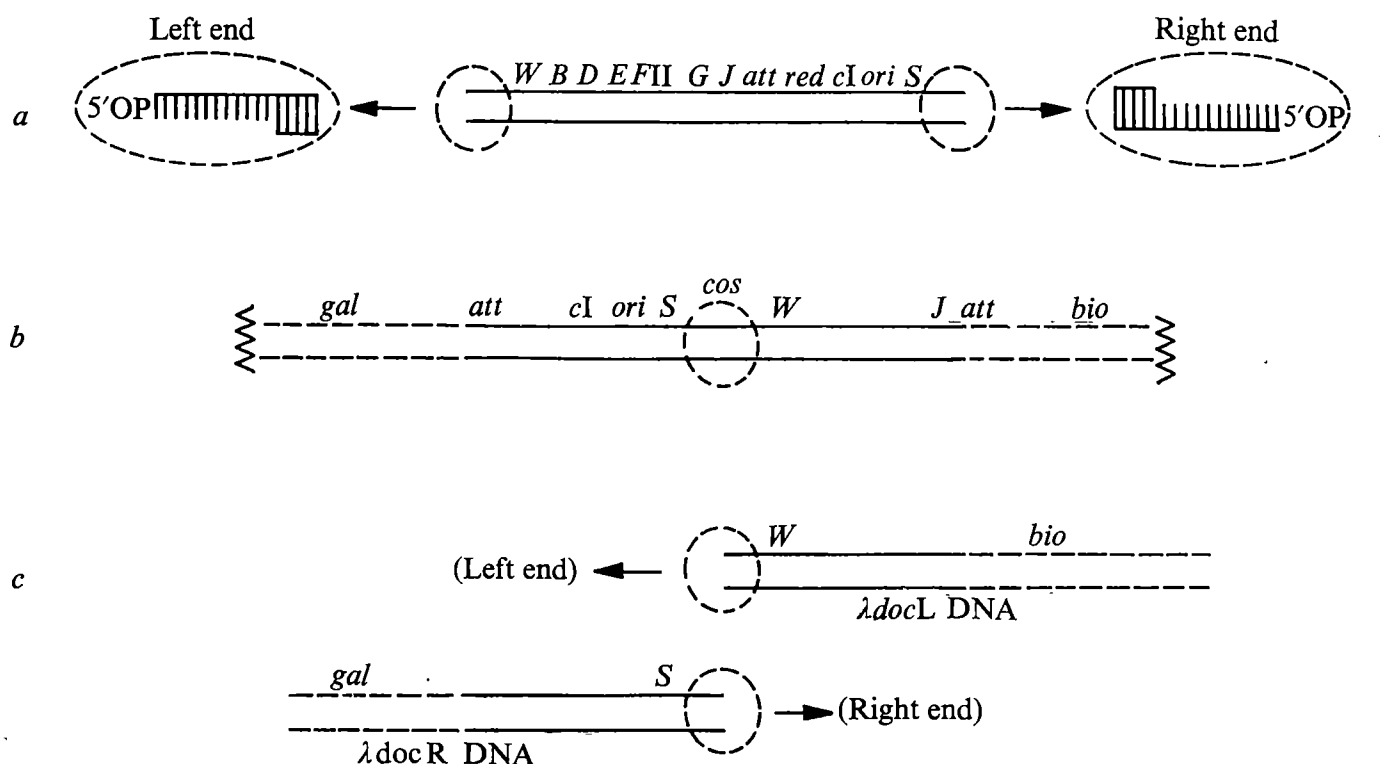
Fig. 1 *a*, Each strand of the DNA of the λ virion is represented by a straight line. The symbols above the double line give the order on the phage genetic map of λ genes mentioned in the text[55]. The distances between genes are arbitrary. The left and right ends of the DNA (within the circles) are shown in expanded scale to the left and right with the vertical bars representing bonds between base pairs, *b*, The inserted prophage and adjoining bacterial DNA are represented according to the same convention except that the bacterial DNA strands are shown by broken lines. Insertion occurs by reciprocal recombination between the site denoted *att* in *a* and another *att* in the bacterial DNA (ref. 26). During insertion the left and right ends of mature DNA are joined by base pairing and ligation[6,26,31]. Consequently the prophage map is a cyclic permutation of the phage map, with the joined ends (within the circle under *cos*) approximately in the centre. *gal* and *bio* are bacterial operons closely linked to the prophage[33]. *c*, Chromosomes of λ*docL* and λ*docR* are represented according to the same convention. The cutting and packaging of these DNAs are described in the text

by covalent bonds[4,5]. This is thought to occur as follows[6-9]. The products of λ genes *A* and *D*, in conjunction with a phage head precursor, make two staggered, single-stranded endonucleolytic cuts 12 base pairs apart at the regions of the polymer corresponding to the mature ends. The sequence-specific cutting activity is called Ter, for terminase, and its site of action is called *cos* (for cohesive end site). The DNA between an adjacent pair of cuts is condensed by an unknown mechanism to approximately 15 times its previous concentration[10] and packaged into a phage head. Cutting and packaging have a common requirement for several phage and at least one host gene[11,12]. Thus, the two reactions are coupled.

DNA cutting by λ differs from that by phage T4 (ref. 13) and phage P22 (ref. 14). Although both of these phages, like λ, cut and package DNA from continuous linear polymers, the length of the mature DNA is determined not by the distance between two specific cutting sites but by the capacity of the head; that is, headfuls of DNA, each slightly larger than the genome, are cut from the polymer. T4 DNA cutting seems to involve no unique recognition site on the DNA. In contrast, mature P22 DNA is formed by sequential cutting and packaging of headfuls from a unique starting site on a polymer[15]. The latter phage, therefore, combines some of the elements of both site-specific and headful cutting.

We report here the production of variant λ particles whose DNA, like that of T4 and P22, is packaged by the headful. These particles are the major product of phage growth when normal packaging is suppressed by the elimination of polymeric λ DNA. In such lysates we also find transducing activity for a large number of bacterial genes. The particles responsible for such activity seem to

be formed by the packaging of headfuls of bacterial DNA in λ capsids.

Packaging of prophage DNA

To examine packaging in the absence of polymeric λ DNA, we characterised the phage particles released after induction of an excision-defective monolysogen. In such a lysogen, phage DNA replication initiates in the prophage at *ori* (Fig. 1*b*) and then proceeds in both directions in the bacterial chromosome[16,17]. The structure formed after several rounds of DNA synthesis have initiated is not known with certainty; it probably resembles a puff of localised replication around the prophage. If so, the prophage replicas would be colinear with an unreplicated prophage (Fig. 1*b*).

How is such DNA packaged? We propose the following model. Ter cuts the prophage at *cos* to form the normal left and right ends of mature λ DNA. DNA to the right of *cos* is preferentially condensed and packaged in phage heads. The amount of DNA per head equals the capacity of the head (a headful). Unpackaged DNA, continuous with the packaged DNA, protrudes from the head, blocking the attachment of tails and preventing the function of λ protein(s) that act at the head–tail junction

Some evidence for this model already exists Gottesman and Yarmolinsky[18] and Little and Gottesman[19] showed that Ter cuts *cos* after induction of an excision-defective lysogen. They also characterised the DNAs of two types of defective phage particles produced· λ*docL* and λ*docR*. λ*docL* DNA carries the left end and λ*docR* DNA the right end of mature λ DNA; both DNAs are colinear with the prophage and in addition carry adjacent bacterial genes (Fig. 1*c*). We have further characterised λ*docL* particles and measured

Table 1 Plaque formers and *bio* transducers produced after induction of an excision-defective monolysogen (strain NS490)

Activity	Units per induced cell		+DNase
	+DNase	−DNase	−DNase
Bio transduction	2×10^{-2}	3.3×10^{-4}	60
Plaque formation	3.8×10^{-5}	3.6×10^{-5}	1.0

The relevant genotype of NS490 is: *supOrecA(λb2red3cIts857 Sam7)*. The excision defect of NS490 is a consequence of inactivation of the right prophage attachment site by the *b2* mutation[18]. A lysate was prepared by heat induction of NS490 as follows. Cells were diluted from a fresh overnight culture into tryptone broth (1 % Difco tryptone, 0 5 % NaCl in distilled water) and grown in a shaking water bath with vigorous aeration at 32 °C to $A_{630} = 0.4$ (this corresponds to 2×10^8 viable cells ml^{-1}). At this point the temperature of the culture was raised rapidly to 42 °C for 15 min and then lowered to 38 °C. After 135 min of incubation at 38 °C the cells were collected by centrifugation, resuspended in 0 02 volume TMG (0.01 M Tris-HCl, *pH* 7 4, 0.01 M MgSO₄, and 0.01 % gelatin) with or without the addition of pancreatic DNase (10 μg ml⁻¹)(Worthington, RNase-free), and incubated with gentle shaking for 30 min at 32 °C together with CHCl₃ (about 0.05 ml ml⁻¹) to promote lysis. The biological activity of such lysates was stable at 4 °C. *Bio* transducers were assayed by infecting a starved overnight culture carrying the *bio*A24 mutation[49] with appropriate dilutions of the above lysate together with five *λcIts857* phage per cell. After 15 min incubation at 32 °C for phage adsorption, a constant number of cells (approximately 10⁸) was added to 2.5 ml molten 0.7% agar in distilled water, and poured into Petri plates containing about 40 ml M9 agar[50] supplemented with 0.5 % glucose, 0.5 % Difco vitamin-free casamino acids, and thiamine (0.1 μg ml⁻¹). Colonies of *bio*⁺ transductants were counted after 24 h incubation at 32 °C. Plaque formers were assayed on strain YMC (*supF*⁺). Plaque formation and the growth of bacterial cultures for transduction were as described[50].

the number produced per cell relative to λ*docR*. The results of these studies are presented next.

Packaging of DNA to the right of *cos*

Our model predicts that an excision-defective lysogen will produce tailless phage heads carrying λ*docL* DNA. In fact, λ*docL* particles, as originally characterised, were uninfective, seemingly because they lacked tails[19] If tail attachment to λ*docL* is prevented by uncut DNA protruding from

Table 2 Requirements for extra cellular maturation of λ*docL* particles

Limiting gene products	Relative infectivity of:	
	λ*docL*	λ*docR*
None	1.0	1.0
J	0.02	1.0
W	0.95	1.0
FII	0.02	1 0

A culture of NS490 was induced and collected as described in Table 1 and then mixed with an induced culture of a second lysogen (see below) in TMG to give about 10⁸ and 10¹⁰ cells per ml, respectively, of the two strains After 10 min incubation at 32 °C in the presence of CHCl₃ (0.05 ml ml⁻¹), DNase (10 μg ml⁻¹) was added and incubation continued for 30 min. The mixtures were then assayed for transducing particles. The numbers in column 2 (λ*docL*) are relative numbers of *bio* transductants (see Table 1). The value 1.0 corresponds to 0 01 *bio* transductants per input NS490 cell When DNase was omitted from the mixture, this value fell to 0.0003 *bio* transductant per NS490. The numbers in column 3 (λ*docR*) are relative numbers of *gal* transductants. A value of 1.0 corresponds to 0.002 *gal* transductants per input NS490. *gal* transduction was measured as described in ref. 50 with strain SA624 (*galT*amA28)[51] as recipient. The second lysogen was always a derivative of strain 594 (*supO strR galK2 galT1*) It was induced and collected as described in the legend to Table 1 for strain NS490. The prophage varied: in line (1), (None), it was λ*Eam4 cIts857 Sam7*, in line (2), (J), λ*Eam4 Jam27 cIts857 Sam7*; in line (3), (W), λ*Wam403 Eam1100 cIts857 Sam7*; and in line (4), (FII), λ*Eam11 01 FIIam423 cIts857 Sam7*. The purpose of the mutations in gene *E*, which encodes the major λ head protein, was to prevent the trapping of other phage proteins in or on head structures made of *E* protein, and to prevent the formation of *bio*-transducing particles by strain 594 lysogens.

the phage head, removal of the excess DNA should lift the block We indeed observed a large increase in infectivity, measured by *bio* transduction, when we treated a λ*docL* lysate with DNase (Table 1, line 1). In contrast, DNase treatment did not increase the infectivity of plaque-forming λ in the same lysate (Table 1, line 2). The latter particles presumably have normal left and right DNA ends as both are required for autonomous phage growth[20,21]. Therefore we do not expect that any DNA will protrude from the head and block tail addition.

If *bio*-transducing particles arise from λ*docL* heads, they will carry the left end of wild-type λ DNA and phage genes from the left arm of the λ chromosome. These predictions are confirmed by the following observations (the data will be presented elsewhere). The *bio*-transducing activity can be concentrated and purified by standard techniques (see Table 4) DNA extracted from purified particles carried the left but not the right end of wild-type λ DNA (ref. 22 and K. Murray, personal communication). The presence of the left end is consistent with the observation that this DNA possessed high transforming activity for *bio* in the transformation assay described by Kaiser and Hogness[23]; such activity requires that the DNA carry at least one λ end[24] This DNA has been restricted by treatment with endonuclease R.*Eco*RI, and the resulting fragments resolved by electrophoresis in agarose gels One of these fragments was indistinguishable in size from that of a 0.44 fractional length fragment cleaved from the left arm of λ DNA by the same enzyme (ref. 25 and D. Tiemeier, personal communication). If the DNA of the *bio*-transducing particles carries only one of the λ ends, it will be unable to circularise and therefore to form a prophage[26]. In fact,
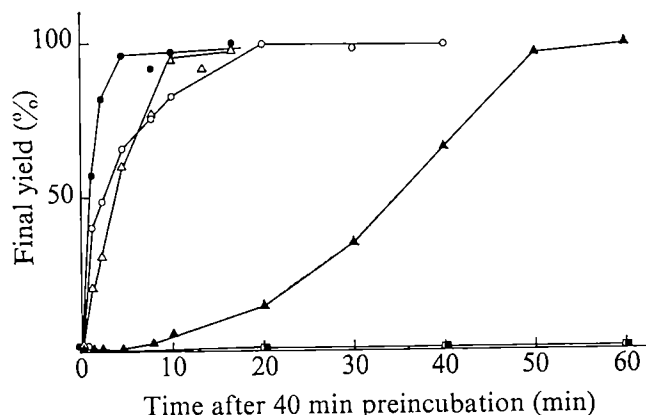
Fig. 2 The kinetics of the *in vitro* completion of λ*docL* virions. λ *doc* particles were produced by induction of *supO recA* (λ*Jam6 b2 red3 cIts857 Sam7*) as described in Table 1, without the addition of DNase. λ tails were produced in the same way by induction of strain 594 (λ*Wam403 Eam1100 cIts857 Sam7*), (see Table 2). Samples (0.2 ml) of the first lysate containing one drop CHCl₃ were preincubated for 40 min at 32 °C in the presence (+DNase) or absence (−DNase) of DNase (0.2 μg ml⁻¹). After this preincubation period (0 min on the figure) the following additions were made to the samples and aliquots were removed at the designated times to assay λ*docL* (*bio* transduction) or λ*docR* (*gal* transduction): ○, (+DNase)+0.2 ml tails, λ*docL* assayed; ■, (−DNase)+0.2 ml tails, λ*docL* assayed; □, (+DNase)+0.2 ml TMG, λ*docL* assayed; ▲, (−DNase)+0.2 ml tails+DNase (0.2 μg ml⁻¹), λ*docL* assayed; ●, (−DNase) +0.2 ml tails+DNase (0 2 μg ml⁻¹), λ*docR* assayed; △, (−DNase)+0.2 ml tails+DNase (10μg ml⁻¹), λ*docL* assayed. The final yield of λ*docL* obtained in the presence of DNase and tails was 0.007-0.008 *bio* transductants per input *recA* cell. This is 70-80 % of that obtained when the λ*doc* lysate, tails and DNase (10 μg ml⁻¹) were mixed immediately after lysis Preincubation of the lysate for 40 min in the presence of tails followed by the addition of DNase (0.2 μg ml⁻¹) gave results identical to ▲. The final yield (0.0007 *gal* transductants per input *recA* cell) and kinetics of formation of λ*docR* were the same in the presence as in the absence of DNase.

Fig. 3 Models for the packaging of prophage and polymeric DNA. *a*, First step in the packaging of prophage DNA is the cutting of *cos* and the encapsulation of DNA to its right by precursor head I together with the products of genes *A*, *D*, *F*I and *W*. The work of several groups shows that the formation of precursor head I (prehead or petit λ) requires the function of phage genes *B*, *C*, *nu*3 and *E* (refs 8, 56 and 57), while the products of genes *A*, *D*, *F*I and *W* act subsequently[7,8,29,40]. All of these genes are required for λ*doc*L production (data not shown). Packaged duplex DNA is shown as a coil; unpackaged duplex DNA as a straight or wavy line. In the lysate, the excess DNA protruding from precursor head II is trimmed by DNase action and the λ*doc*L particle is completed by the addition of *F*II protein and tails. *b*, Packaging of polymeric DNA differs from that of the prophage in that adjacent copies of *cos* are both cut, and the intervening DNA is packaged. Therefore, no excess DNA protrudes from the head, and *F*II protein and tails add intracellularly. We show DNA associated with the tail of λ but not with that of λ*doc*L (J. Thomas, N.S. and R.W., unpublished).

less than 1% of the *bio* transductants were lysogens. We have observed cotransduction of the *bio*A gene with λ left phage arm genes *B*, *D*, and *J* by using *bio*A recipients that carried suitably marked prophages. In the light of these observations, we refer here to the *bio*-transducing particles as λ*doc*L particles.

The hypothesis that functional tails add to λ*doc*L heads only after extracellular DNase treatment is confirmed by the following experiment. An induced excision-defective lysogen was diluted 100-fold with a second induced lysogen that carried a λ*J*⁻ prophage, and the mixture was lysed by adding chloroform followed by DNase. Only the excision-defective prophage can synthesise tails[27,28]; thus, we predict that the formation of infective λ*doc*L should be limited by the low concentration of tails in this mixture. In fact, the number of *bio* transducers was 30-fold less than that found when the excision-defective lysogen was diluted with a tail-proficient lysogen (Table 2, lines 1 and 2). In contrast, the infectivity of phage particles whose DNA carried a wild-type λ right end (λ*doc*R; Fig. 1c,) was unaffected by dilution with a tail-defective lysogen (Table 2). These observations show that the attachment of functional tails to λ*doc*L heads occurs extracellularly, whereas attachment to

λ*doc*R heads occurs intracellularly. Is this also true of λ proteins that act at the head–tail junction? To answer this question we diluted the λ*doc*L-producing lysogen before lysis with a *W*-defective or an *F*II-defective lysogen. The protein products of the *W* and *F*II genes are known to act sequentially on DNA-filled heads to permit the attachment of tails[29]. After lysis of the mixtures, we found normal levels of *bio*-transducing activity in the *W*-deficient but not in the *F*II-deficient mixture (Table 2, lines 3 and 4). We conclude that *W* but not *F*II product can act on λ*doc*L heads intracellularly.

The above conclusions are consistent with the effect of varying the order of addition of tails and DNase on the subsequent rate of formation of infective λ*doc*L. Figure 2 shows that if tails were added to a tailless λ*doc* lysate before or at the same time as DNase, there was a delay in the formation of infective λ*doc*L relative to λ*doc*R. The delay was eliminated when DNase was added to the lysate before tails. We conclude that DNA must be digested before functional tails can add to λ*doc*L heads. We suspect this is also true for the action of *F*II product.

Our model of the formation of λ*doc*L is compared with the formation of λ in Fig. 3.

Capacity of head and asymmetry of packaging

The amount of DNA per λdocL particle that remains after DNase treatment should correspond to the capacity of the λ head (Fig. 3). This prediction can be verified by equilibrium density gradient centrifugation of a DNase-treated λdocL lysate, as the density of a λ particle (and presumably of a λdocL particle) is a known function of its DNA content[1]. The results of such an experiment (Fig. 4) show that the density profile of bio-transducing activity had a peak corresponding to a DNA content 104% that of wild-type λ and a width suggesting some heterogeneity in the amount of DNA per particle. This is about the maximum amount of DNA that can be packaged in a λ head without a reduction of phage growth: a phage mutant with a DNA content of 109% had a burst size only 10% that of wild-type λ, and this reduction is directly attributable to the increased DNA size[30]. A mutant with a DNA content of 106.5% (λimm434att[2]) had a larger but still reduced burst (N.S. and R.W., unpublished).

In addition to λdocL, an excision-defective lysogen produces another particle, λdocR, whose DNA consists of the right arm of the λ chromosome and bacterial DNA to the left of attλ. It is formed by the packaging of prophage and bacterial DNA to the left of cos[19] (Fig. 1c). λdocR can be physically separated from λdocL by equilibrium density gradient centrifugation (see ref 19 for the density distribution of λdocR). We found that the number of λdocL particles in a lysate exceeded that of λdocR by a factor of 200–600 (Table 3, column 2). The great excess of λdocL relative to λdocR confirms our hypothesis that DNA to the right of cos is packaged preferentially. Data to be presented elsewhere suggest that this asymmetry is a consequence of asymmetry in cos (N.S. and R.W., unpublished).

How is λdocR DNA cut from the bacterial chromosome? At present, we can only offer a tentative answer to this question. The right end of λdocR DNA must be formed by Ter acting specifically at cos; the left end may be formed through nonspecific cutting by Ter or by some other intra-cellular nuclease. Little and Gottesman[19] have shown that the left end is not at a specific site. The DNase independence of λdocR infectivity (data not shown) suggests that the left end is formed intracellularly.

While measuring the number of particles in λdoc lysates, we found that the ratio of infective to total particles (the specific infectivity) of λdocR was 100–200 times greater than that of λdocL (Table 3, column 4). This is at least partly explained by the defective DNA injection of λdocL particles (J. Thomas, N.S. and R.W., unpublished) We shall consider this point further below.

Generalised transducing particles

Lysates of an induced excision-defective lysogen also contained particles that transduced many different bacterial genes (Table 4). A detailed analysis of these particles will be presented elsewhere (N.S. and R.W., unpublished); we list here some of their salient properties. Transduction was abolished if the lysate was treated with anti-λ serum or if the bacterial recipient was unable to adsorb λ. Therefore, the activity is associated with λ virions Generalised transducers were found in lysates of recA bacteria infected with λ int⁻red⁻. Therefore, neither prophage insertion[41] nor any of the major recombination pathways of phage or host[32] is required. In fact, the production of generalised transducing particles was reduced by the action of the Red recombination pathway. This observation is not understood. All the transductants examined were haploid and carried no phage markers. This suggests that transduction occurs exclusively by substitution of infecting DNA for homologous recipient DNA. Transduction by substitution is expected if the transducing DNA is unable to circularise[26]. The transducing activity for markers close to attλ was

lower in lysates made by infection than in those made by induction. We attribute this to preferential replication of bacterial genes near the prophage. The transducing activity of a lysate made by infection varied over a 100-fold range for different markers. This suggests that certain regions of the bacterial chromosome are preferentially packaged by λ. Certain pairs of closely linked bacterial markers—such as argH-rif and gal-bio (ref. 33)—were cotransduced by λ. The appearance of generalised transducing activity, like λdocL infectivity, required DNase treatment of the lysate (Table 4) in the presence of FII protein and tails (data not shown). The density distribution of transducing activity for five of six markers examined coincided precisely with that of λdocL (see Fig. 4; his was the exception).

How are generalised transducing particles formed? The following hypothesis, although difficult to test, is appealing because of its simplicity. We assume the existence of sites in the bacterial chromosome whose nucleotide sequence resembles that of cos. These sites are cut with low efficiency
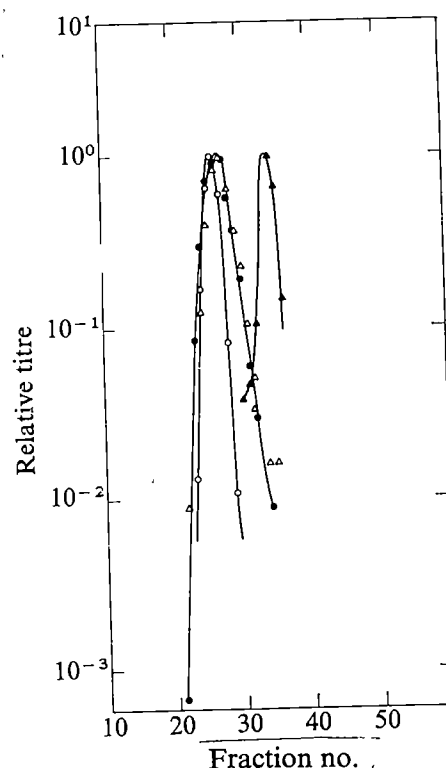
Fig. 4 A CsCl equilibrium density gradient of λdocL. A DNase-treated lysate of strain NS490, prepared as described in Table 1, was partially purified by low speed centrifugation (6,000g for 10 min at 4 °C) followed by centrifugation of the supernatant on a CsCl step gradient for 60 min at 4 °C in a Beckman SW56 rotor. (Four steps of densities 1.7, 1.55, 1.45 and 1.30 g ml⁻³ were used[58].) Fractions were collected from the bottom of the tube and those containing most of λdocR and λdocL infectivity were pooled and dialysed against 0.1 M Tris-HCl, pH 7.4, 0.01 M MgSO₄; 5 μl of this was mixed with 2.484 g solid CsCl and 3.03 ml TMG containing λatt[2] int am29 imm434 cIts1 (ref. 59) and λb2 immλ cI (ref. 18). The DNA content of the former phage is 106.5% (ref. 1 and M. Fiandt, et al. unpublished) and the latter 88% (ref. 1) that of λ. These phage were centrifuged for 22 h at 35,000 r.p.m. in a Beckman SW50 rotor at 4 °C. Sixty-two fractions were collected from a hole punctured in the bottom of the tube and assayed for λdocL (●) (bio transducers; Table 1); trp transducers (△), (using strain C224 trp (λ)[60] as host; Table 4); λatt[2] (○); and λb2 (▲), (turbid and clear plaque formers, respectively, on the non-lysogenic strain C600, plates incubated at 32 °C). The λdocR activity (Table 3) was found in a broad peak within fractions 30–37 (data not shown). Densities were related to DNA contents by the equation of Bellett et al.[61]. In a separate experiment, A₂₆₀ of the fractions was measured as well. The absorbance profile coincided with that of λdocL, the predominant λdoc species (Table 3).

Table 3 Yield per cell of λ*doc*L and λ*doc*R

Phage	Units per induced cell (a) Total particles	(b) Infective particles	Specific infectivity (b / a)
λ*doc*L	4	0.02	0.005
λ*doc*R	0.01–0.02	0.01	0.5–1

A DNase-treated lysate of NS490 derived from 300 l induced cells was prepared as described in Table 1 except that the initial concentration factor was 1,000-fold rather than 50-fold Bacterial debris was removed by low-speed centrifugation, and the phage were sedimented by overnight centrifugation at 6,000g at 4 °C. The pellet was resuspended in 30 ml TMG, and the phage purified by centrifugation first through a CsCl step gradient and then in a CsCl equilibrium gradient. The latter step separates λ*doc*L from λ*doc*R (see Fig. 4). λ*doc*R was assayed by plaque formation on strain N585 (ref. 19), [*sup*0 (λ*Nam*7 *imm*434; 21*hy*5)]. This assay, which depends on recombination between the *imm*λ marker of λ*doc*R and the right arm of the prophage, is about two to fourfold more efficient than *gal* transduction (data not shown). λ*doc*L was assayed by *bio* transduction (see Table 1). To measure total particles, the CsCl equilibrium gradient fractions containing most of the λ*doc*L and λ*doc*R infectivity were pooled separately and dialysed against TMG.A_{260} of each pool was measured and the values obtained converted to numbers of physical particles by assuming that an $A = 1.0$ corresponds to 3×10^{11} plaque-forming units per ml wild-type λ (ref. 52), and that the specific infectivity of wild-type λ is 1.0 The number thus obtained for λ*doc*L was confirmed by comparing the serum blocking power[53,54] of the pooled λ*doc*L with that of λ*cIts857 Sam7* purified in the same way. (The titration of neutralising antiserum with λ virions presumably measures the concentration of tail fibres.) Identical results were obtained with a second 300 l preparation and, for λ*doc*L, with 20 ml lysate labelled with ^{3}H-thymine

by Ter, and adjacent DNA to the right is packaged according to the model we have proposed for λ*doc*L. At present we have insufficient evidence to distinguish this model from one that assumes nonspecific cutting either by Ter or by another nuclease. In addition, it remains to be shown that the transducing particles contain exclusively bacterial DNA. Nevertheless, the coincidence between the density distribution of most of the transducing activity and that of λ*doc*L strongly suggests that λ packages bacterial DNA by the headful in these conditions.

Discussion

How are the packaging of prophage and polymeric DNA related? Our models of the two processes (Fig 3) differ in one important respect: precursor head II, the DNA of which is continuous with unpackaged DNA, is unique to prophage packaging. We know little about the properties of this intermediate and are attempting to confirm its existence by electron microscopic examination of purified preparations. Because of the higher concentration and consequent lower entropy of packaged DNA, we might expect precursor head II to be unstable. On the contrary, it seems to be stable for at least 40 min in a concentrated lysate (Fig. 2). (This conclusion rests on the assumption that a constant fraction of the precursor head II present in concentrated lysate is converted to *bio*-transducing particles by treatment with DNase.) In contrast, similar structures formed during DNA packaging by phages T4 and T7 (prohead III (ref. 34) and the 40S structure[35], respectively) seem to be unstable. This difference may reflect a difference in experimental conditions or a difference in the precursors.

Our results indicate that the prophage DNA to the right of *cos* is packaged more efficiently than DNA to the left. The results of Emmons[36] demonstrated an asymmetry in the packaging of polymeric DNA. He explained this by proposing that packaging initiates at *cos* and proceeds rightwards. This proposal is completely consistent with our findings.

We find that the yield of λ*doc*L particles from an induced excision-defective single lysogen is four per cell (Table 3). This suggests that *cos* is cut at least once in every cell.

Freifelder *et al.*[37] have proposed that two copies of *cos* on a single molecule are required for optimum Ter cutting. Our measurements of the total yield of λ*doc*L do not support this proposal: the yield is no greater in a tandem polylysogen than in a single lysogen even though the production of plaque-forming particles is much higher in the former strain (N.S. and R.W., unpublished). We are measuring the kinetics of λ*doc*L production in the two strains.

The failure of tails to add to precursor head II is not surprising in view of the intimate and specific association between the tail of the wild-type virion and the right end of mature DNA (refs 38 and 39). Incubation of precursor head II with DNase in the presence of *F*II protein and tails markedly increases the infectivity of λ*doc*L (Table 1). Nevertheless, fewer than 1% of the total particles are infective (Table 3). The uninfective particles lack neither tails nor *F*II protein. The presence of tails and therefore of *F*II protein[29,40] is shown by: the concordance of the serum-blocking power with the A_{260} of purified λ*doc*L (see legend to Table 3); the concordance of the CsCl equilibrium density profile of the absorbance with that of the *bio*-transducing activity (see legend to Fig. 4); and electron microscopic observations of the particles adsorbed to bacteria (J. Thomas, N.S. and R.W., unpublished). Therefore, an explanation of the low specific infectivity of λ*doc*L must be sought elsewhere. We find that it results, at least in part, from inefficient DNA injection by tailed particles, and we postpone further discussion to a later paper.

Many bacteriophage species mediate generalised transduction. For some we know that the transducing particles contain little or no phage DNA (ref. 41, 42); apparently these phages can cut and package the bacterial DNA. We suspect that λ generalised transducing particles also contain exclusively bacterial DNA, although this has not been proved. If this DNA is packaged according to the model proposed earlier, only one Ter cut in the bacterial chromosome is needed for each particle. The absence of generalised transduction when DNase is omitted can then be explained by the improbability of finding in the bacterial chromosome two Ter-specific cutting sites that are separated by the length range compatible with phage viability (about 36,000–51,000 base pairs[30,43]).

How do other phages that normally contain DNA with specific ends package bacterial DNA in the absence of DNase? For phage P22 the answer may lie in the model of DNA packaging proposed by Tye *et al.*[15], elaborated by Chelala and Margolin[44]: only the first cut in the bacterial chromosome need be specific; other, nonspecific cuts can

Table 4 Production of generalised transducing particles

Marker transduced	Units per induced cell +DNase	−DNase
*glt*A, *nad*A, *aro*A	10^{-3}	$< 10^{-5}$
*pur*E, *pyr*D	10^{-4}	$< 10^{-6}$
*trp*E, *pro*A	2×10^{-5}	$< 2 \times 10^{-7}$
thr, *leu*, *arg*H, *pyr*C	$2{-}4 \times 10^{-6}$	$< 10^{-8}$
his	5×10^{-7}	$< 10^{-8}$

DNase-treated and untreated lysates of strain NS490, prepared as described in Table 1, were assayed for transducing activity on the following hosts: AB1157, obtained from the NIH collection, for *arg*H, *pro*A, *his*, *leu* and *thr*, X9170 and MP005, obtained from Dr W. Epstein, for *glt*A and *nad*A, respectively; AB478 and MSO, obtained from Dr S. Adhya, for *aro*A and *pyr*D, respectively; X5214, obtained from Dr E. Signer, for *pyr*C; X821, obtained from Dr R. Curtiss III, for *pur*E; and Y *mel trp* E5947, obtained from Dr C. Yanofsky, for *trp*E. All strains not already lysogenic for λ were lysogenised to reduce killing by plaque-forming phage present in the lysate. Transduction was performed as described for *bio* (see Table 1) except that no coinfecting λ was added, and vitamin-free casamino acids were omitted from the plating agar for transduction of *glt*A, *nad*A, *pur*E, *pyr*D, *pro*A, *thr*, *leu*, *arg*H, *pyr*C and *his*.

be made by a nuclease that is associated with the phage head In the case of phage Mu-1 (ref. 45) one end of the phage DNA is known to be variable, both in nucleotide sequence and in length[46]. It is therefore possible that a sequence-specific nuclease cuts one end of both phage and transducing particle DNA, while the other end is cut non-specifically. Generalised transduction by phage T1 (ref. 47) is more difficult to explain Normal T1 DNA is linear with a duplex terminal repetition[48] The extent of the repetition varies slightly in extent at both ends of the DNA (ref. 48). This imprecision in the ends suggests that the T1 DNA cutting mechanism may be more tolerant than that of λ to sequence variability. Alternatively, the production of T1 generalised transducing particles may require a nuclease not normally used to cut the phage DNA.

We thank Drs D. Botstein, M. E. Gottesman and J. Weil for suggestions; and Drs L. Enquist, H. Nash and M. Shulman for criticising the manuscript.

1 Davidson, N , and Szybalski, W , in *The Bacteriophage* λ (edit. by Hershey, A. D.), 45-82 (Cold Spring Harbor, New York, 1971).
2 Wu, R., and Taylor, E., *J molec Biol* , 57, 491-511 (1971).
3 Hershey, A , and Burgi, E , *Proc. natn. Acad Sci. U.S A* , 53, 325-328 (1965).
4 Skaka, A , Poonian, M , and Bartl, P., *J. molec. Biol* , 64, 541-550 (1972)
5 Wake, R., Kaiser, A , and Inman, R., *J. molec , Biol.*, 64, 519-540 (1972).
6 Yarmolinsky, M., in *The Bateriophage* λ (edit. by Hershey, A. D), 97-111 (Cold Spring Harbor, New York, 1971).
7 Hohn, B., and Hohn T., *Proc natn. Acad Sci U.S A.*, 76, 2372-2376 (1974).
8 Hohn, B , Wurtz, M., Klein, B., Lustig, A., and Hohn, T., *J supramolec Struct.*, 2, 302-317 (1974)
9 Kaiser, D., Syvanen, M , and Masuda, T., *T molec Biol* , 91, 175-186 (1975)
10 Kellenberger, E., and Edgar, R. S., in *The Bacteriophage* λ (edit by Hershey, A.D.), 271-295 (Cold Spring Harbor, New York, 1971)
11 MacKinlay, A , and Kaiser, A , *J molec. Biol* , 39, 679-683 (1969).
12 Georgopoulos, C. P., Hendrix, R. W., Casjens, S , and Kaiser, A D., *J molec. Biol* , 76, 45-60 (1973).
13 Streisinger, G , Emrich, J , and Stahl, M. M., *Proc. natn Acad Sci. U S.A.*, 57, 292-295 (1967).
14 Tye, B , Chan, R K., and Botstein, D , *J molec. Biol* , 85, 485-500 (1974)
15 Tye, B , Huberman, J A , and Botstein, D., *J. molec. Biol.*, 85, 501-532 (1974)
16 Stevens, W., Adyha, S , and Szybalski, W., in *The Bacteriophage* λ (edit. by Hershey, A. D), 515-533 (Cold Spring Harbor, New York, 1971)
17 Imae, Y., and Fukasawa, T , *J. molec. Biol* , 54, 585-597 (1970).
18 Gottesman, M., and Yarmolinsky, M., *Cold Spring Harb. Symp. quant. Biol.*, 33, 735-748 (1968)
19 Little, J , and Gottesman, M., in *The Bacteriophage* λ (edit. by Hershey, A. D.), 371-394 (Cold Spring Harbor, New York, 1971).
20 Kayajanian, G., *Virology*, 36, 30-41 (1968).
21 Padmanabhan, R., Wu, R , and Bode, V C., *J. molec. Biol.*, 69, 201-207 (1972).
22 Murray, K., and Murray, N , *Nature new Biol* , 243, 134-139 (1973).
23 Kaiser, A. D, and Hogness, D., *J molec. Biol.*, 2, 392-415 (1960).
24 Kaiser, A. D., and Inman, R., *J. molec. Biol* , 13, 78-91 (1965).
25 Allet, B , Jeppesen, K., Katagiri, K., and Delius, H., *Nature*, 241, 120-122 (1973).
26 Campbell, A , *Adv Genet* , 11, 101-145 (1962).
27 Weigle, J., *Proc natn. Acad. Sci. U S.A.*, 55, 1462-1466 (1966).
28 Kuhl, P., and Katsura, I., *Virology*, 63, 221-237 (1975)
29 Casjens, S , Hohn, T., and Kaiser, A. D., *J. molec. Biol.*, 64, 551-563 (1972).
30 Weil, J., Cunningham, R., Martin, R., Mitchell, E . and Bolling, B., *Virology*, 50, 373-380 (1972)
31 Gottesman, M , and Weisberg, R , in *The Bacteriophage* λ (edit.by Hershey, A.D.), 113-138 (Cold Spring Harbor, New York, 1971).
32 Signer, E., in *The Bacteriophage* λ (edit. by Hershey, A.D.), 139-174 (Cold Spring Harbor, New York, 1971)
33 Taylor, A., and Trotter, C., *Bact. Rev* , 36, 504-524 (1972).
34 Laemmli, U., and Favre, M., *J. molec Biol.*, 80, 575-599 (1973).
35 Serwer, P., *Virology*, 59, 89-107 (1974).
36 Emmons, S., *J molec. Biol.*, 83, 511-525 (1974)
37 Freifelder, D., Chud, L., and Levine, E E., *J. molec Biol.*, 83, 503-509 (1974)
38 Thomas, J., *J. molec. Biol.*, 87, 1-9 (1974)
39 Chattoraj, D., and Inman, R., *J. molec. Biol.*, 87, 11-22 (1974).
40 Casjens, S., *J. molec. Biol.*, 90, 1-23 (1974).
41 Ikeda, H., and Tomizawa, J., *J. molec. Biol.*, 14, 89-109 (1965).
42 Schmieger, H , *Molec. gen Genet.*, 109, 323-337 (1970).
43 Bellett, A., Busse, H , and Baldwin, R., in *The Bacteriophage* λ (edit. by Hershey, A. D.), 501-513 (Cold Spring Harbor, New York, 1971).
44 Chelala, C., and Margolin, P., *Molec. gen Genet.*, 131, 97-112 (1974).
45 Howe, M., *Virology*, 55, 103-117 (1973).
46 Daniell, E , Abelson, J , Kim, J , and Davidson, N , *Virology*, 51, 237-239 (1972).
47 Drexler, H., *Proc natn Acad. Sci. U S.A.*, 66, 1083-1088 (1970).
48 MacHattie, L., Rhoades. M., and Thomas, C., *J. molec. Biol.*, 72, 645-656 (1972).
49 Del Campillo-Campbell. A., Kayajanian, G., Campbell, A , and Adhya, S , *J. Bact.*, 94, 2065-2066 (1967).
50 Shimada, K., Weisberg, R., and Gottesman, M , *J. molec. Biol.*, 63, 483-503, (1972)
51 Adhya, S., and Shapiro, J., *Genetics*, 62, 231-247 (1969).
52 Bode, V., and Kaiser, A , *J. molec. Biol* , 14, 399-417 (1965).
53 Dove, W., *J. molec Biol.*, 19, 187-201 (1966)
54 Katsura, I., and Kuhl, P., *J. molec. Biol* , 91, 257-263 (1975).
55 Szybalski, W., and Herskowitz, I., in *The Bacteriophage* λ (edit. by Hershey, A.D.), 778-779 (Cold Spring Harbor, New York, 1971)
56 Hendrix, R., and Casjens, S., *J. molec Biol.*, 79, 187-199 (1975).
57 Ray, P., and Murialdo, H , *Virology* (in the press)
58 Caro, L , and Berg, C., *Meth. Enzym* , 21, 444-458 (1972)
59 Shulman, M., and Gottesman, M , in *The Bacteriophage* λ (edit by Hershey A. D), 447-488 (Cold Spring Harbor, New York, 1971)
60 Cleary, P., Campbell, A., and Chang, R , *Proc. natn. Acad. Sci. U.S.A.*, 69, 2219-2223 (1972).
61 Bellett, A., Busse, H., and Baldwin, R., in *The Bacteriophage* λ (edit. by Hershey, A D.), 501-513 (Cold Spring Harbor, New York, 1971).

Two genes in the major histocompatibility complex control immune response

A. J. Munro & M. J. Taussig

Immunology Division, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

Antibody production in mice is controlled by two functionally distinct gene types in the I region of the major histocompatibility complex. One controls T cell recognition of antigen and T cell mediators of cell cooperation, and the other controls the B cell response to T cell mediators.

THE immune response to various antigens is under the controlling influence of genes in the major histocompatibility complex (MHC), termed immune response or *Ir* genes[1-3]. These genes are probably present in all vertebrates including man[4,5], where they may influence the outcome of various diseases[5,6]. The responses controlled by these genes are dependent on thymus-derived lymphocytes or T cells, and include cell-mediated immune responses (for example delayed hypersensitivity) and thymus (T)-dependent antibody responses. A major feature of the MHC-linked *Ir* genes is that they show apparent antigen specificity, though they are clearly separate from the genes coding for serum antibody[1-3]. We have been concerned mainly with the role of *Ir* genes in T-dependent antibody responses, especially in the cooperative interaction which is required between T cells and bone-marrow derived lymphocytes (B cells) for the latter to be triggered to antibody production.

In the past, attention has focused on whether the *Ir* genes code for the antigen-recognition system of T cells—that is the T cell antigen receptor[1-3], a structure which is known to exist but which has proved very hard to characterise. A problem with this interpretation of *Ir* gene function has been that in certain experiments—including our own—the locus of the action of *Ir* genes seems to be in B cells[7-10]. These contradictions can be reconciled if there are two kinds of *Ir* gene controlling the response to each antigen, one gene expressed in T cells and the other in B cells, with different functions in each cell type. We have developed assays for the products of the *Ir* genes and found strong evidence to support a two-gene model.

T cell factor

Our experiments have depended on the production by T cells of an antigen-specific molecule termed a T cell 'factor' which is capable of replacing T cells in thymus-dependent antibody responses[11]. Figure 1 summarises the production and assay of this factor. We have shown that the factor carries a binding site for antigen by absorption with antigen-coated columns. Furthermore, the factor is not an immunoglobulin—it has a molecular weight of about 50,000, and does not react with anti-immunoglobulin reagents. On the other hand, the factor

Preparation of antigen-specific factor

Test of antigen-specific factor
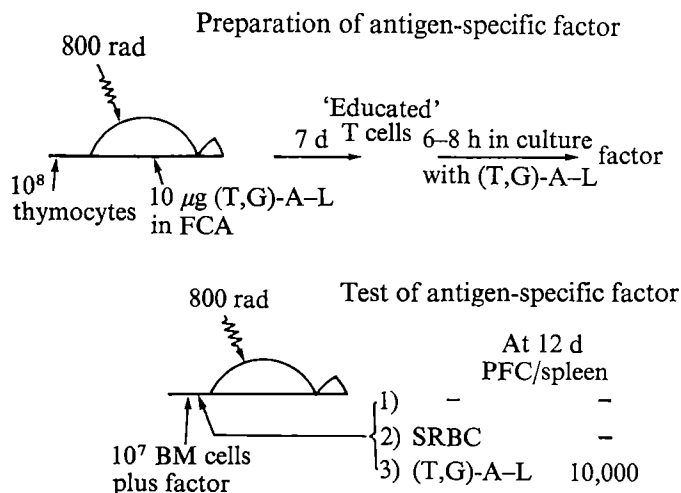
Fig. 1 Preparation and test of antigen-specific T-cell factor. The factor was prepared from mouse thymocytes which had been specifically primed or 'educated' to a synthetic polypeptide antigen such as (T,G)-A-L (poly (Tyr, Gln)-poly DL Ala-poly Lys)[17,18]. The educated mouse T cells were cultured for 6-8 h with antigen *in vitro*, then removed by centrifugation. The culture supernatant was the source of T cell factor. Its activity was assayed *in vivo* by transfer together with bone marrow cells and antigen, (T,G)-A-L, into a lethally irradiated recipient syngeneic with the bone marrow donor. 12-14 d later the spleens of these animals were found to contain specific antibody producing cells against (T,G)-A-L which can be enumerated as plaque-forming cells (PFC). Factor produced against (T,G)-A-L will not stimulate a response against an unrelated antigen such as sheep red cells (SRBC)[11].

does react with antisera raised against the major histocompatibility complex of the mouse (*H*-2), and using region-specific antisera we have mapped the genes involved to the *I* region of *H*-2[12,13]. These properties suggest that the T cell factor is the soluble expression of the T cell antigen receptor, and that the latter is coded for, at least in part, by the *I* region of *H*-2. Work from other laboratories supports this view. For example, a very similar antigen-specific molecule—though functionally suppressive—has been described by Tada and coworkers[14,15]; in addition, other T cell factors, though lacking antigen specificity, are also products of the *I* region, and probably closely related to the specific T cell factors[3,16].

Detection of B cell acceptor

Since the *I* region contains the *Ir* genes, the T cell factor is an obvious candidate for a product of these genes. Ability to produce the factor could therefore be used as an assay for *Ir*-gene function, and it would be predicted that in at least

Table 1 Responses of high responder and low responder bone marrow cells to (T,G)-A-L and T cell factor in F_1 recipients

T cell factor*	Bone marrow cells	Log_{10} PFC/spleen
No†	High responder (B10)	1 311
Yes	High responder (B10)	4 623
Yes	Low responder (B10.A)	1.462
Yes	F_1 (B10×B10.A)	4.724
	Standard error	0.184

*Factor specific for (T,G)-A-L was prepared in B10 BR (*H*-2^k) mice, low responders.

†Control: bone marrow cells transferred into irradiated recipients with antigen but without factor.

Responses given by 10^7 bone marrow cells when combined with 10 μg (T,G)-A-L) and the (T,G)-A-L specific T cell factor, and transferred into irradiated F_1 (B10×B10.A) recipients The bone marrow cells were of either high responder (B10), low responder (B10.A) or F_1 origin. Direct anti-(T,G)-A-L PFC measured in spleens of recipients 14 d after transfer. Results as log_{10} geometric means, standard error shown.

some cases, low responders would lack the ability to make the T-cell specific factor. When this was investigated using as antigen the synthetic polypeptide (T,G)-A-L[17,18], for which high and low responders are known it was found that a low responder strain (C3H/HeJ, *H*-2^k) produced the cooperative T cell factor as efficiently as high responders (C3H.SW, *H*-2^b). On the other hand, the factor, whether of high or of low responder origin, would only cooperate effectively with bone marrow cells of high responder origin[10]. From these experiments, it was concluded that the defect in low responders (of *H*-2^k haplotype) was expressed in the function of bone marrow cells rather than T cells. Table 1 shows an experiment to confirm this result. In this experiment, F_1 irradiated animals (B10×B10.A) were used as recipients for either high responder (B10 or F_1) or low responder (B10.A) bone marrow cells, which were transferred with the same T cell factor and antigen. It is seen that only the high responder bone marrow cells responded. This experiment also serves to rule out possible contributions of the irradiated recipients to the result, since identical F_1 (responder) recipients were used throughout.

In summary, *Ir* genes can be expressed in bone marrow cells, where they might function to receive the T cell product. It has already been predicted that B cells carry an 'acceptor' site for T cell factors[19], and it seems likely that this could be where the bone marrow defect is localised. A direct assay for

Table 2 Absorption of T cell factor by high and low responder bone marrow cells

T cell factor*	Bone marrow cells used for absorption†	Log_{10} PFC/spleen
No‡	—	1.261
Yes	—	4.230
Yes	High responder (B10)	1.799
Yes	Low responder (B10 A)	4.518
	Standard error	0.330

*Factor specific for (T,G)-A-L was prepared in B10.BR (*H*-2^k) mice.

†Factor sufficient for five recipients was absorbed with $5×10^7$ bone marrow cells.

‡Control: response in absence of factor

T cell factor for (T,G)-A-L was absorbed with either high or low responder bone marrow cells, and any remaining factor was mixed with F_1 (B10×B10 A) bone marrow cells and (T,G)-A-L and transferred into irradiated F_1 recipients. Direct PFC to (T,G)-A-L were measured 14 d later in the spleens of the recipients. Results as log_{10} geometric means.

the presence of an acceptor site on bone marrow cells is measurement of the ability of these cells to absorb T cell factor. As shown in Table 2, high responder (B10) bone marrow cells completely absorb the factor activity; low responder (B10.A) bone marrow cells, in contrast, do not absorb the factor at all. Similar experiments showed that the origin of the factor (that is, high or low responder) did not affect the result and that factor could also be absorbed in the absence of antigen. These experiments provide strong evidence for the existence of an acceptor in high responder, but not in certain low responder, bone marrow cells. Since bone marrow contains diverse cell types we have begun experiments with purified cell populations. Our preliminary results show that the acceptor is present on peripheral B cells (anti-θ-treated lymph node cells depleted of macrophages); the presence of an acceptor on macrophages and peripheral T cells has yet to be established. ·

It was clearly important for the interpretation of these experiments to show that the factor was taken up by B cells in a biologically meaningful way. This was demonstrated by transferring bone marrow cells which had adsorbed factor into lethally irradiated recipients with antigen but without further addition of factor. It was found that provided the adsorption

had occurred in the presence of the specific antigen, the bone marrow cells made a good response on transfer. Presumably the antigen was necessary to focus factor on to the antigen-specific B cells.

The absence of the acceptor site from the B cells of a low responder to (T,G)–A–L, indicated that *Ir* genes might directly code for the acceptor. Table 3 shows that functional binding of factor to the acceptor site can be blocked by anti-H-2 sera. Bone marrow cells of F_1 origin (high×low responder: B10× B10.A) were treated with anti-H-2 sera before absorbing factor in the presence of antigen. Anti-H-2[b] (that is, antibody to the high responder haplotype) prevented the bone marrow cells from functionally absorbing the factor, as measured by the response of the bone marrow cells in irradiated recipients, whereas anti-H-2[a] (the low responder haplotype) did not block the acceptor. Thus the acceptor seems to be coded for by genes in the *H-2* complex, and experiments are in progress to determine whether it is an *I*-region product, as predicted.

In summary, we have identified two products of the *H-2* complex, both probably coded by the *I* region, which are involved in T–B cell cooperation, namely the T cell factor and the B cell acceptor. As described above, there exist strains which are low responders to (T,G)–A–L because they lack the B cell acceptor. Do all non-responders share a B cell defect, or do some strains have T cell deficiencies which take the form of failure to make the specific cooperative factor? We have screened several strains for their ability to make T cell specific factor and for their B cell acceptor function. Table 4 summarises the results, and shows that while several strains have only B cell defects, one strain (B10.M, *H-2[f]*) has a 'pure' T cell defect, and another (SJL, *H-2[s]*) lacks both T and B cell function[32]. These findings make it very likely that two *Ir* genes are involved in control of the antibody response to (T,G)–A–L, one expressed in T cells, the other expressed in B cells. In order to confirm the two-gene hypothesis, we have studied the response of F_1s between non-responders of complementary type (that is, T or B cell deficient). As shown in Table 5, such F_1s are high responders. This complementation shows that almost certainly two genes affect the antibody response to specific antigens. Since in one F_1 combination (B10.BR×B10.M) the mice were congenic resistant and differ only at *H-2*, both the genes are located in the *H-2* complex, and from what has gone before, probably both in the *I-A* subregion. Backcross experiments are in progress to confirm this.

Taken together, the results described here provide strong evidence that two functionally distinct genes in the MHC determine the level of T-dependent immune responses by their effect on cell interaction. Other workers have found evidence for two *Ir* genes in the MHC by analysis of F_1 hybrids[20-22].

Table 4 Cellular defects in antibody response to (T,G)–A–L

H-2	Strain	Response	T cell factor	B cell response
b	B10, C3H.SW	High	Yes	Yes
d	B10.D2, BALB/c	High	Yes	Yes
k	B10.BR, C3H/HeJ	Low	Yes	No
q	DBA/1	Low	n.d	No
j	I.St	Low	Yes	No
f	B10.M	Low	No	Yes
s	SJL	Low	No	No

Data from refs 10, 12, 32 and unpublished.

For example, Rüde and Günther found complementation in the response to (T,G)–A–L in rat F_1 hybrids[21], and more recently Benacerraf and colleagues have shown that two *Ir* genes control the response to the synthetic polypeptide GLPhe[11] in mice[22]. In the latter case, the genes were shown to map in different *I* subregions. It seems reasonable to expect that at least two MHC-linked genes will be found to determine the response levels to each thymus-dependent antigen. Our experiments take these findings further by defining the mode of action of the genes and their products. Thus one type of *Ir* gene codes for the antigen-specific T cell regulators of the immune response, and by inference, the T cell antigen recognition system. The second type of gene, by coding for an acceptor for the T cell regulators on lymphocytes determines the ultimate response of the cell to antigen (Fig. 2).

Model accounting for antigen specificity

We must now consider how the two genes would incorporate the antigen specificity which is the remarkable feature of the genetic control of the immune response. In the case of T cell defects in response an obvious possibility is that failure to respond to an antigen is due to the absence of the specific binding site for that antigen from the repertoire of T cell antigen receptors. The presence of *Ir* genes expressed in the B cell, however, requires a more complex explanation for the apparent antigen specificity of *Ir* gene control.

A model to account for the findings is as follows: (1) We propose that there must be several 'classes' of T cell factor,

Table 3 Blocking acceptor with anti-H-2 sera

Bone marrow cells	Factor*	Antiserum pretreatment of bone marrow cells†	Log$_{10}$ PFC per spleen
F_1 (B10×B10.A)			
	Yes	—	2.857
	Yes	Anti-H-2[b]	1.397
	Yes	Anti-H-2[a]	3.230
		Standard error	0.250

*Specific for (T,G)–A–L, prepared in B10.D2 (*H-2[d]*) mice.
†5×10^{7} bone marrow cells in 1 ml medium were heated with 0.1 ml undiluted antiserum for 30 min at 4 °C and then washed. Factor and (T,G)–A–L were then added and incubated with the cells for a further 30 min. The cells were again washed and then transferred into five irradiated F_1 recipients Antisera used were B10.D2 anti CWB (anti-H-2[b]) and B10 anti A/J (anti-H-2[a]).

T cell factor specific for (T,G)–A–L was absorbed, in the presence of (T,G)–A–L, on to either normal or antiserum-treated F_1 (B10× B10.A) bone marrow cells. The cells were then transferred, without further addition of factor, into irradiated F_1 recipients. The direct anti-(T,G)–A–L PFC responses in the spleens of the recipients were measured 12 d later. Results as log$_{10}$ geometric means.

Fig. 2 Cell interaction in the antibody response Model showing the possible relationship between T cell receptor, specific T cell factor and B cell acceptor in cell cooperation and a mechanism of B cell triggering. 'Ia' refers to the antigenic determinants carried by the T cell factor detected by antisera raised against the *I* region. The *Ir* genes are believed to code for the T cell receptor, the T cell factor and the B cell acceptor
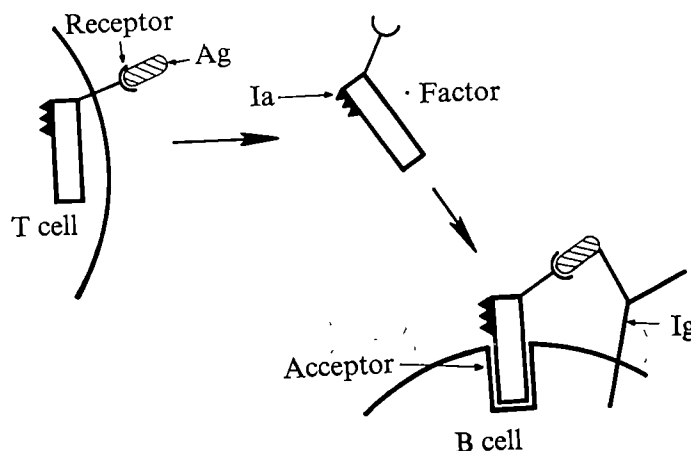
Table 5 Antibody response to (T,G)–A–L in F_1 hybrids of low responder strains

Strain	H-2	Response (PFC per spleen)
B10*	b	4.162
I.St	j	1 681
B10.BR	k	1.342
B10.M	f	1 079
(I.St×B10.M)F_1	j/f	3.875
(B10 Br×B10.M)F_1	k/f	4 291
	Standard error	0 362

*Control high responder

Primary antibody responses, measured as direct anti-(T,G)–A–L PFC, 14 d after inoculation of 10 μg (T,G)–A–L in complete Freund's adjuvant, intraperitoneally. Log_{10} geometric means.

analogous in a sense to the classes of immunoglobulin and that for each factor class there exists a corresponding acceptor. The genes for a T cell factor class and the corresponding acceptor for that class comprise a 'set'. Note that the T factor is assumed to be clonally expressed, whereas each B cell would carry all classes of acceptor site. (2) Within each set there would be genes which contribute to the antigen-binding sites carried by that class of factor. Taking again the analogy with immunoglobulin genes, each set would contain a number of 'variable' (V) genes which could become associated with the 'constant' (C) gene for the T factor of that set (Fig. 3). (3) An important point is that the V genes of any one set will only code for part of the total binding site repertoire of T cell factors. In this way there will be antigens which will only react with the binding sites of one set, although there will be other antigens for which several sets are available. (4) The loss of a B cell acceptor for one class of T factor will render the B cell incapable of response to factor of that class, and thus to any antigen which is dependent solely on that set for cell cooperation. This would predict that all antigens which are restricted to that defective set would fail to give responses. (5) Two types of T cell defect are possible, namely (a) a defect in a specific antigen binding site, as already mentioned, and (b) a defect in the constant region of the T cell factor, leading to the effective inactivation of the entire set. (6) A consequence of the model is that 'Ir-gene' control will only be noticeable for antigens which are restricted to the binding sites of one set. This explains the well-known observation that genetic control is in general only observed for antigens where a limited number of determinants are presented to the
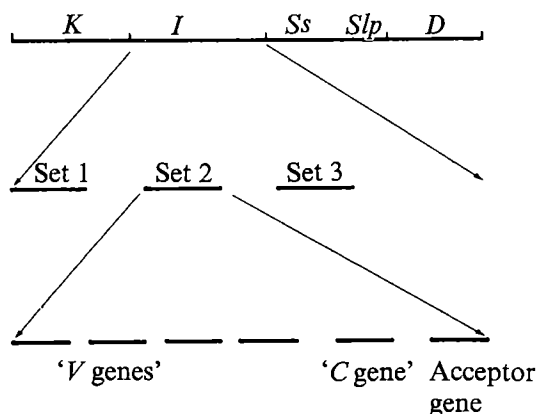
system, for exampe synthetic polypeptides, proteins in limiting dose, and alloantigens[1]. (7) The different patterns of response which antigens show in different strains[1-3,23], arise in part because of the different genetic causes of low responsiveness. Furthermore, there is the possibility of genetic rearrangements, so that the genes comprising each set can vary between strains.

Although this model explains a great many of the phenomena associated with Ir genes, there are certain observations which require further discussion. For example, while we have shown that T cell factor can be produced to (T,G)–A–L in certain low responder strains, these same strains (notably H-2^k) lack other specific T cell-dependent activities such as delayed hypersensitivity (Davies and Shearer, personal communication), and proliferation of T cells in response to antigen in vivo[24], and in vitro[25]. This dichotomy could be explained if T cell–T cell interaction were necessary for the generation of effector T cells, and if this interaction took place by the same molecular mechanism as T cell–B cell cooperation. Alternatively, suppressor T cells may be generated in situations where low response results from an acceptor defect[26,27]. A further problem arises from the response to (T,G)–A–L in tetraparental mice constructed from high responder (H-2^k) parents, where low responder B cells produced antibody to (T,G)–A–L[28]. This is at first sight difficult to reconcile with our demonstration of an acceptor defect on H-2^k B cells. But it may be that conditions in the responding tetraparental create an environment in which the acceptor defect can be bypassed. Alternatively strains nominally of the same H-2 haplotype may have undergone rearrangement or mutation of genes in the I region. The locus of unresponsiveness in the animals used to contruct the tetraparentals would have to be determined to distinguish these possibilities.

Finally, the emerging significance of the I-region products, not only in genetic control, cell cooperation and factor production, but also in mixed lymphocyte reaction[29], graft-*versus*-host response[30], immunological enhancement[31], and so on, suggests that these phenomena may all be reflections of the same molecular structures.

Fig. 3 Immune response genes of the H-2 complex. Schematic representation of the possible arrangement of genes in 'sets' in the I region of H-2 Within each set are genes coding for the binding site of the T cell receptor and factors 'V genes', for the 'constant' region of the receptor and factors 'C genes', and for the acceptor

1 Benacerraf, B , and McDevitt, H. O., *Science*, 175, 273–279 (1972)
2 *Genetic Control of Immune Responsiveness* (edit by McDevitt, H O., and Landy, M.) (Academic, New York and London, 1972)
3 Benacerraf, B , and Katz, D H , in *Immunogenetics and Immunodeficiency* (edit. by Benacerraf, B) (Medical and Technical, London, in the press).
4 Levine, B. B , Stember, R. H., and Fotino, M , *Science*, 178, 1201–1203 (1972).
5 *Transplant. Rev.*, 22 (1975).
6 McDevitt, H. O , and Bodmer, W. F., *Lancet*, i, 1269–1275 (1974).
7 Mozes, E , and Shearer, H G., *Curr. Top. Microbiol. Immun* , 59, 167 (1972).
8 Katz, D. H., Hamaoka, T , Dorf, M E , Maurer, P H., and Benacerraf, B , *J exp Med* , 138, 734–739 (1973)
9 Mozes, E , *Progr. Immun* , II, 2, 197–201 (1974).
10 Taussig, M J , Mozes, E , and Isac, R., *J. exp Med.*, 140, 301–312 (1974)
11 Taussig, M J , *Nature*, 248, 234–236 (1974).
12 Munro, A. J , Taussig, M J , Campbell, R , Williams, H , and Lawson,Y., *J exp Med.*, 140, 1579–1587 (1974)
13 Taussig, M J , Munro, A J , Campbell, R., David, C S , and Staines, N A , *J exp. Med* (in the press)
14 Tada, T., Okumura, K., and Taniguchi, M , *J Immun.*, 111, 952–961 (1973)
15 Tada, T., in *Immune Recognition Proc 9th Leuk. Cult. Conf* (edit. by Rosenthal, A) (Academic, New York, in the press)
16 Armerding, D , Sachs, D. H , and Katz, D. H , *J. exp. Med* , 140, 1717–1722 (1974).
17 Fuchs, S , and Sela, M , in *Handbook of Experimental Immunology*, 1, Chapter 11 (edit by Weir, D H) (Blackwells, Oxford, 1973)
18 Sela, M., *Harvey Lecturers Series*, 67, 213–246 (1973)
19 Katz, D. H., Hamaoka, T., Dorf, M. E , and Benacerraf, B , *Proc. natn Acad. Sci U S A.*, 70, 2624–2628 (1973)
20 Zaleski, M , Fuji, H , and Milgrom, F , *Transplant Proc* , 5, 201–204 (1973)
21 Rude, E , and Gunther, E., *Prog Immun* , 11, 2, 223–233 (1974)
22 Dorf, M. E , Stimpfling, J H , and Benacerraf, B., *J exp. Med.* (in the press).
23 Benacerraf, B., and Katz, D H., *Adv. Cancer Res* , 21 (in the press)
24 Hammerling, G J , and McDevitt, H O , *J. Immun* , 112, 1734–1740 (1974)
25 Lonai, P , and McDevitt, H. O , *J exp. Med* , 140, 977–994 (1974)
26 Gershon, R K , Maurer, P H , and Merryman, C F , *Proc. natn. Acad. Sci. U S A.*, 70, 250–254 (1973).
27 Kapp, J. A., Pierce, C. W , Schlosseman, S , and Benacerraf, B , *J exp Med* , 140, 648–659 (1974).
28 Bechtol, K B , Freed, J. H , Herzenberg, L A , and McDevitt, H O., *J. exp Med* , 140, 1660–1675 (1974)
29 Bach, F H., Widmer, H B , Bach, M L., and Klein, J , *J. exp Med* , 136, 1430–1444 (1972).
30 Klein, J., and Park, J M , *J. exp Med.*, 137, 1213–1225 (1973).
31 Staines, N. A., Guy, K., and Davies, D A. L., *Eur J Immun* (in the press)
32 Mozes, E., Isac, R., and Taussig, M J., *J exp. Med* , 141, 703–707 (1975)

letters to nature

X-ray observations of Cyg X-1 with ANS

FROM May 1 to May 8, 1975, Cyg X-1 was observed with the instruments on board, the Astronomical Netherlands Satellite (ANS). We report here the results of the measurements performed with soft and hard X-ray detectors of the Space Research Laboratory in Utrecht and the Center for Astrophysics in Cambridge, USA. Cyg X-1 underwent an upward transition in its intensity[1], that seems to be the inverse of the downward transition seen by Uhuru in April 1971. The bulk of the increase occurs at low energy: between 1 and 2 keV, the intensity increased by a factor of 10 over the November 1974 intensity observed by ANS, while above 8 keV there is no significant change.

The Utrecht experiment consists of two detectors. A 1.7 μm titanium window proportional counter of 45.0 cm^2 effective area collimated to a field of view of 36'×90' (FWHM) is sensitive in the energy range 0.35–0.45 keV and 1–7 keV, divided over 6 pulse height channels. The second instrument, a parabolic reflector with a projected area of 144 cm^2, has in its focus a small-area counter with a window of 3.6 μm polypropylene. The field of view is a 34' circle. A full description of the instrument is given in ref. 2.

The relevant portion of the Cambridge experiment consists of 50 μm beryllium window proportional counters of 50 cm^2 effective area collimated to a field of view of 10'×3° (FWHM) sensitive in the energy range 1.3–30 keV divided into 15 energy channels[3].

The light curves of Cyg X-1, as observed in the medium energy range (1–7 keV) of the Utrecht detector and in the 1.3–7 keV window of the Cambridge detector, are presented in Fig. 1. The source is observed to be in a high intensity state, similar to the one observed[4] before the transition in April 1971. The count rate has increased by a factor of 8–10 in the Utrecht detector and a factor of about 4 in the Cambridge detector compared with the observations on Cyg X-1 made with ANS in November 1974 (our unpublished work with W. Forman, C. Jones and

Y. Tanaka). The count rate in the first measurement on May 1 is at the lowest point for both detectors, at 80 counts s^{-1} and 58 counts s^{-1} respectively, and rises to 100 counts s^{-1} (80 counts s^{-1}) on May 3, suggesting that the source is in its last stage of the actual transition. After May 3, the intensity averages above 100 counts s^{-1} (80 counts s^{-1}) with excursions up to 160 counts s^{-1} (130 counts s^{-1}). The spectrum is in all data very steep, similar to the one observed[4] before April 1971. In fitting the measured pulse height spectrum with a source spectrum of the form

$$dF/dE = AE^{-\alpha}\exp(-\sigma n_H) \ \text{keV}(\text{cm}^2 \text{ s keV})^{-1}$$

where σ is the Brown and Gould[5] interstellar absorption and E the photon energy in keV, we find a best fit for the first measurement on May 1 of $\alpha = 2.6 \pm 0.2$ and n_H (equivalent) $= 7^{+3}_{-1} \times 10^{21}$ atoms cm^{-2}. In general, all the measurements fit with a power-law energy index range in the narrow band of 2.2–2.8, with an absorption of $8^{+4}_{-2} \times 10^{21}$ atoms cm^{-2}, whereas in the November 1974 data the spectrum was as flat as $\alpha = 0.4$, changing on a time scale of minutes to $\alpha = -0.5$ (a positive slope). The beryllium window counters also show little if any change in the intensity and spectrum above 8 keV, as compared to the ANS observations of November 1974.

The polypropylene counter–parabolic mirror combination has a transmission window between 0.2 and 0.28 keV and a side lobe between 0.4 and 0.7 keV. The pulse height discriminator levels are optimised for 0.2 to 0.28 keV, and in view of detector resolution set to 0 13 and 0.41 keV. The small efficiency at 0.4–0.7 keV is due to the finite counter resolution in the 0.13–0.41 keV pulse height interval. By integrating the Cyg X-1 signal in the parabolic section over 1,600 s, we observe a count rate of 0.38±0.04 counts s^{-1}, which in view of the large column density of interstellar matter to the source must be due to the high energy end of the detector window. The interstellar absorption for 7×10^{21} atoms cm^{-2}, assuming Brown and Gould[5]
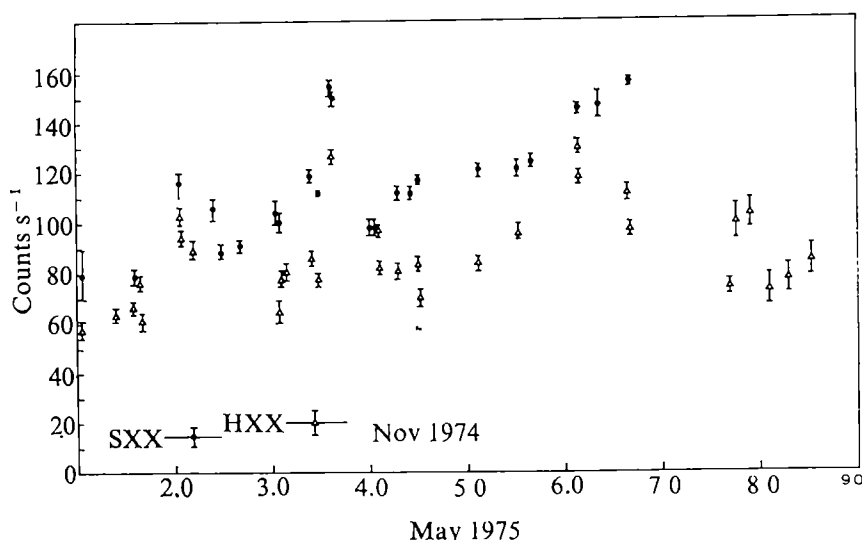
Fig. 1 The preliminary light curve of Cyg X-1 between May 1 and May 8, 1975. Black dots indicate the count rate of the Utrecht medium energy detector SXX (1–7 keV), open triangles are the count rates of the Cambridge hard X-ray detectors HXX (1.3–30 keV). The average count rate during the November 1974 observations are also indicated. Bars indicated typical excursions in intensity during observations of a few minutes. Marker on date axis indicates 0000 UT for indicated day.
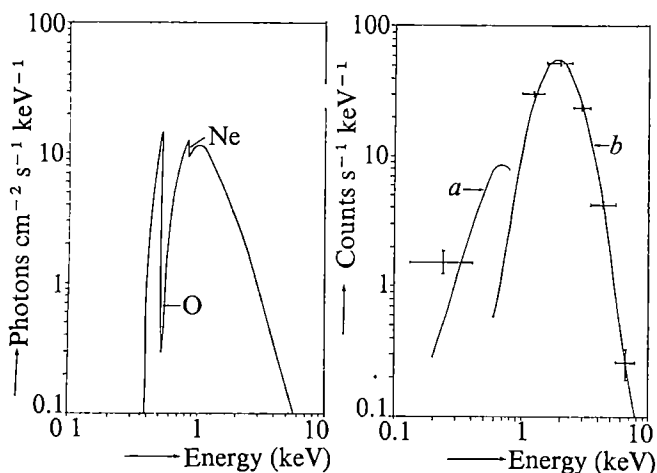
Fig. 2 Spectrum of Cyg X-1. The left-hand side shows the flux at Earth for a typical set of best fit parameters. O and Ne mark the absorption edges. The right-hand side shows this spectrum convolved with the detector response of the two Utrecht detectors and the observed count rates in the different pulse height channels. The signal in the polypropylene counter (*a*) is integrated over a longer time interval than that of the Ti counter (*b*).

abundances, is 3.3 mean free paths just below the oxygen absorption edge of 0.53 keV, and 19 mean free paths at 0.28 keV. The ultraviolet flux of the ninth magnitude BOI stellar companion of Cyg X-1 is three orders of magnitude too low to explain the soft flux by contamination of the ultraviolet flux, both from the theoretical point of view and from the measured ultraviolet spectrum[6]. The detected flux in the soft X-ray counter is entirely consistent with an assumed source spectrum with power law (energy) index of 2 5 and interstellar absorption of 7×10^{21} atoms cm^{-2}. The expected count rate for this spectrum is $0.28 \ s^{-1}$. This spectrum is presented in Fig. 2, together with the measured flux and the spectrum convolved with the response of the two detectors. With this spectrum the source intensity at 0 50 keV then corresponds to 13 ± 3 photons $cm^{-2} \ s^{-1} \ keV^{-1}$ and the power spectrum is valid over the entire range from 0.5 to 6 keV (ref. 7).

The intensity and spectrum of X radiation from Cyg X-1 observed by the ANS instruments are now dramatically different from those observed by the same instruments during November 1974. The present data, both the high intensity and the changed spectra, are consistent with what was reported by Uhuru for the 'high' state of 1970–71. For this reason, and because this high level has persisted for about six days as we see it, we conclude that Cyg X-1 has reverted to its high state. Our data also suggest that the transition was in progress when we began observing on May 1.

Several descriptions have been put forward to explain the observed low and high intensity states of Cyg X-1 on the basis of accretion disk models[8]. In these models the flux above 10 keV originates in the optically thin inner disk, and the flux below 10 keV in the optically thick region immediately surrounding the inner disk. The transition could be due to a change in accretion rate or to the switch on and off of a secular (Lightman and Erdlich) instability in the inner region of the accretion disk[9] where the disk could become spatially thick and optically thin wherever radiation pressure dominates gas pressure. Thorne and Price[8] make various predictions for the photon energy E_{max} at which the source spectrum exhibits a maximum.

From the spectrum presented in this paper $E_{max} < 0.5$ keV suggesting, on the basis of the qualitative model of Thorne and Price, that the transition is triggered by a change in

the accretion rate in a disk model where the optically thin region is indeed extended (Lightman–Erdlich instability operative).

The power spectrum of Cyg X-1 integrated from 0.5 to 6 keV leads to an intrinsic luminosity at 2.5 kpc ranging between 7.0×10^{37} and 9.6×10^{37} erg s^{-1}.

Extended X-ray, optical and radio measurements are now in progress and may help clarify the nature of this transition. On May 9 the radio emission was several times higher than at any time in the previous four years[10]. This is in contrast to the behaviour during the previous high state when Cyg X-1 was not seen in radio. Also, Bolton[11] has not seen any spectroscopic changes in the optical emission.

This work was supported in part by NASA.

J. HEISE   R. MEWE
A. C. BRINKMAN   A. DEN BOGGENDE
J. SCHRIJVER   E. GRONENSCHILD

Astronomical Institute,
Space Research Laboratory,
Beneluxlaan 21,
Utrecht, The Netherlands

D. PARSIGNAULT

American Science and Engineering,
955 Massachusetts Avenue,
Cambridge, Massachusetts 02139

J. GRINDLAY   E. SCHREIER
H. SCHNOPPER   H. GURSKY

Center for Astrophysics,
60 Garden Street,
Cambridge, Massachusetts 02139

1 Gursky, H., *et al*, *IAU Circ. no* 2778, May 9 (1975).
2 Brinkman, A. Ch., Heise, J , and de Jager, C , *Philips tech Rev*, 34, 43 (1974).
3 Gursky, H., Schnopper, H., and Parsignault, D. R., *Proc. Cospar Symp. X and Gamma Ray Astronomy, Astrophys. Space Sci* (in the press)
4 Tananbaum, H , Gursky, H., Kellogg, E., Giacconi, R , and Jones, C , *Astrophys. J. Lett*, 177, L5 (1972).
5 Brown, R. L., and Gould, R. J , *Phys. Rev D*, 1, 2252 (1970)
6 Wu, C. C., Wesselius, P. R., van Duinen, R. J., De Boer, K. S , and Aalders, J W. G., *IAU Circ no* 2779, May 15 (1975).
7 Heise, J , Brinkman, A C , Mewe, R., Schrijver, J., Den Boggende, A. F. F , and Gronenschild, E., *IAU Circ. no* 2779, May 15 (1975).
8 Thorne, K. S , and Price, R H , *Astrophys J , Lett*, 195, L101 (1975)
9 Lightman, A P., and Eardley, D M , *Astrophys. J., Lett.*, 187, L1 (1974)
10 Hjellming, R. M., Gibson, D. M., and Owen, E. N., *IAU Circ no.* 2779, May 15 (1975).
11 Bolton, C. T , *IAU Circ no* 2779, May 15 (1975).

Observations of a new transition in the emission from Cyg X-1

THE all-sky X-ray monitor aboard Ariel V is a pinhole camera sensitive to X rays in the energy range 3–6 keV. It has an effective area of 0.6 cm^2 with a duty cycle of $\sim 1 \%$ for a given source, and monitors more than 3π of the celestial sphere each orbit[1].

Following the first report[2] that the emission from Cyg X-1 had increased, we used data from the all-sky monitor to establish that a sudden transition had occurred on April 22, 1975, 9 d before the initial observation from ANS. Cyg X-1 has been observed almost continuously since $\sim$ November 1, 1974, by this experiment, and has been relatively constant in apparent magnitude until the reported effect. Its pre-transition average intensity was 0.5 ± 0.1 efficiency-corrected photons $cm^{-2} \ s^{-1}$ in the energy range 3–6 keV, where the 20% error is the estimated absolute calibration uncertainty. During the > 5 months before the transition, the average daily intensity was
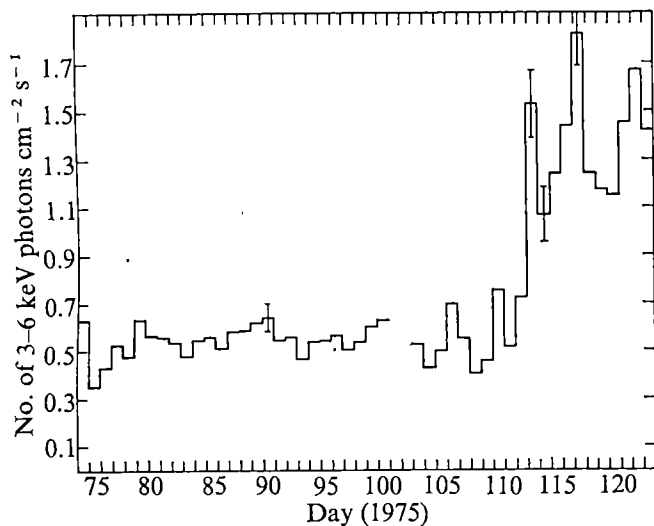
Fig. 1 Daily average intensity of Cyg X-1 for 50 d. The constancy of the intensity before day 112 is representative of the source (as measured by this experiment) since November 1974.

rarely measured as high as 0.7 $cm^{-2} s^{-1}$. The Crab Nebula intensity in the same units is 1.4 $cm^{-2} s^{-1}$.

Daily averages between March 15, 1975 (day 74), and May 3, 1975 (day 123), are displayed in Fig. 1. No Cyg X-1 data were taken during days 101 and 102, but the data taken up to day 100 are representative of the behaviour of Cyg X-1 since the onset of experimental operation. From days 103–123 Cyg X-1 was located at approximately the same spacecraft latitude, so that no intensity changes may be ascribed to systematic errors in the data analysis. On day 123 the satellite was reoriented to allow the four Ariel-V experiments which view along the spin axis to observe Cyg X-1, placing it outside the field of view of this experiment.

The data after day 103 are shown with an approximately half-day resolution in Fig. 2. The natural resolution of the experiment is one satellite orbit ($\sim$100 min), but the statistical errors on some data points then become more than half as large as the magnitudes, so that we have not attempted to present finer resolution than that shown in Fig. 2.

Clearly, the rise above 1 $cm^{-2} s^{-1}$ during the latter half of day 112 is real, as the intensity continues to rise to more than three

Fig. 2 Intensity measured from Cyg X-1 over approximately half-day accumulation times. The source is no longer in the field of view of the experiment after day 123 (May 3).
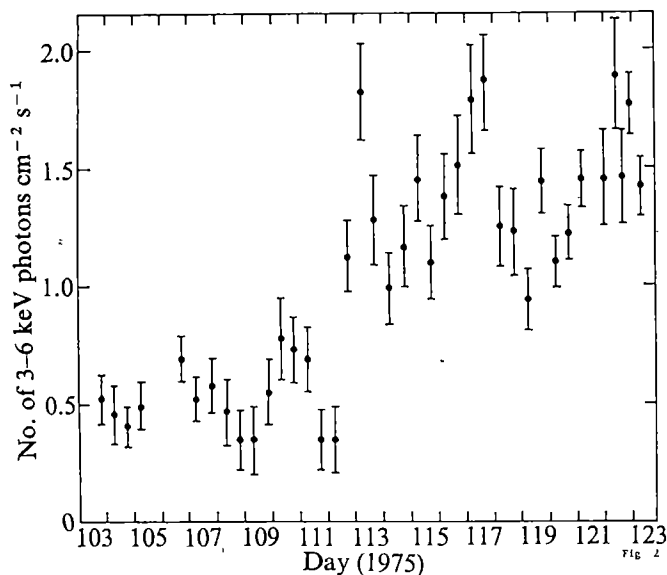
times the pre-transition average value during the first half of day 113. The intensity then seems to oscillate between $\sim$1 and $\sim$2 $cm^{-2} s^{-1}$ with a time scale of several days until it passes out of our field of view on day 123 (May 3, 1975). As the experiment has no spectral resolution, we cannot comment on any spectral change which may have accompanied the intensity transition. The 3–6 keV acceptance window is relatively insensitive to spectral shape, however, so that the indicated intensities in the 3–6 keV band should be correct whether or not the spectrum changes.

Cyg X-1 has remained remarkably constant in intensity and spectrum[3] since the "transition" in 1971 (ref. 4). Before 1971, the intensity was extremely variable, and the spectrum was always softer than that measured afterwards. Whether or not the effect reported here represents a long term return to its pretransition state is something which this experiment will be able to monitor in the future.

Millisecond temporal structure in the emission from this source has previously been measured only by large area rocket-borne experiments[5], and the present intensity increase should now make possible its study with instruments of smaller area mounted on various satellites. L.J.K. acknowledges support from the University of Maryland.

S. S. HOLT
E. A. BOLDT
L. J. KALUZIENSKI
P. J. SERLEMITSOS

Laboratory for High Energy Astrophysics,
NASA/Goddard Space Flight Center,
Greenbelt, Maryland 20771

1 Holt, S. S., *Proc. Cospar Symp. Fast Transients X and gamma ray Astronomy, Astrophys. Space Sci.,* (in the press).
2 Gursky, H., *et al, IAU Circ. no.* 2778 (1975).
3 Rothschild, R. E, Boldt, E. A., Holt, S. S., and Serlemitsos, P. J., *Bull. Am. Phys Soc.,* 20, 604 (1975).
4 Tananbaum, H , Gursky, H., Kellogg, E., Giacconi, R., and Jones, C , *Astrophys. J. Lett.,* 177, L5 (1972).
5 Rothschild, R. E , Boldt, E. A., Holt, S. S , and Serlemitsos, P. J., *Astrophys. J. Lett.,* 180, L13 (1974).

Ariel V and Copernicus measurements of the X-ray variability of Cyg X-1

THE collimated proportional counter (experiment C of MSSL) on Ariel V viewed Cyg X-1 in 1975 from May 3 1400 UT until May 15 1000 UT. We report here observations of the intensity and spectrum obtained with an integration time of $\sim$30 min. They show that, at the beginning of this period, Cyg X-1 was more intense, had a steeper spectrum and was generally more active than has been observed during the past 2½ yr by Copernicus[1,2]. Towards the end of the period the source seemed to be returning to its lower state.

Figure 1 shows the 1.5–15 keV and the 2–6 keV fluxes from Cyg X-1 during the Ariel V observations. The dominant errors in the values of intensity are due to uncertainty in the total integration time, rather than statistical errors, and it is the total $\pm 1\sigma$ errors which are shown on the two intensity light curves. Also plotted against time is the photon index of the power law spectrum obtained by fitting the data in the 1.5–15 keV energy range. The values observed (with Ariel V) during a brief look in October 1974 are also indicated.

The intensity and spectral index were indicative of a high state[4] at the start of the observation on May 3, 2 d after the onset of the low–high transition reported[3] from ANS. The source remained in the high state until May 9 or 10; it then began to recover its low state values represented by the October 1974 data. The transition back to

Table 1	2-10 keV X-ray observations of Cyg X-1			
Date	Measurement	Ref	Photon power law spectral index	State of source
June 1964	Rocket (NRL)	5	Not given	High (based on intensity)
April 1965	Rocket (NRL)	6	Not given	Low (based on intensity)
October 1965	Rocket (ILL)	7	Not given	Low (based on intensity)
October 1966	Rocket (AS&E)	8	-1.7 ± 0.1	Low
September 1967	Rocket (NRL)	9	-1.5 ± 0.1	Low
September 1970	Rocket (GSFC)	10	-2.6 ± 0.3	High
November 1970	Rocket (MSSL)	11	-2.8 ± 0.2	High
December 1970 to March 1971	Uhuru	4	-4.05 ± 0.14	High
April 1971 to May 1972	Uhuru	4	-1.45 ± 0.03	Low
October 1972 to September 1974	Copernicus	1, 2 and this paper	-1.6 ± 0.1	Low
October 1974	Ariel V	This paper	-1.7 ± 0.2	Low
May 1975	ANS	14	-3.5 ± 0.2	High
May 1975	Ariel V	This paper	-4.2 ± 0.2 to -2.1 ± 0.2	High, then approaching low
May 1975	Copernicus	This paper	-2.1 ± 0.2	Approaching low

the low state was not complete when the satellite moved on in its observing programme on May 15.

Figure 2 shows the measured spectrum and best fit power laws of two typical orbits at the beginning and end of this run of data. The spectrum of May 6 shows an excess of high energy X rays above the power law spectrum, as indicated by the higher value of χ^2.

The high and low states seen by Ariel V are similar in intensity and spectral index to those reported from Uhuru[4] before and after the down transition about April 1, 1971. The high energy excess in Fig. 2a (in the high state) is also seen in the Uhuru data before this transition, but the Ariel V spectrum does not have the sharp change of slope reported from Uhuru[4].

Copernicus has observed Cyg X-1 on ten occasions between November 6, 1972, and May 12, 1975, of which the first seven are summarised by Mason et al.[2]. A measurement of spectral index on May 12, 1975, by Copernicus agrees with the value -2.1 ± 0.2 measured then by Ariel V. On all the nine previous occasions the slope was significantly different at -1.6 ± 0.1.

The implication is that Cyg X-1 was in its low state from April 1, 1971, until May 1, 1975, and in a high state for about 20 d thereafter. (If, however, 20–30 d is a typical duration for the high state, such flashes could slip between gaps in the Copernicus coverage, which are up to 180 d long.)

Table 1 lists spectral indices in the energy range 2–10 keV of Cyg X-1 taken from the literature. The first measurement of a spectral index considerably different from $\alpha = -1.6$ was reported by Bleach et al.[10] on September 21, 1970. Subsequently data from Uhuru[4] also yielded a departure from the usual value of the spectral index, as did a measurement by Cruise[11], made on November 11, 1970, which showed $\alpha = -2.8$. It is possible that this high state, characterised by enhanced emission in the 2–10 keV region, together with a steeper and more variable spectrum, lasted at least six months until the down transition reported by Tananbaum et al.[4]. It is improbable but not impossible for an up state of this duration to have occurred during the Copernicus measurements without having been detected. It seems that the low state, characterised by the power law spectral index stable at -1.6, is the usual state of Cyg X-1 in which the source remains for about 90% of the time.

We have searched the Copernicus data taken between 1972 and 1975 for any systematic change in X-ray behaviour which might herald a transition. One possibility is a variation in the orbital phase at which absorption events are seen[2]. The data of Mason et al.[2] suggest a systematic change in phase of the absorption events on a time scale of a few years (their Fig. 3), and this trend is confirmed by two further observations: Li and Clark[12] observed, with OSO 7, an absorption event in June 1973 at
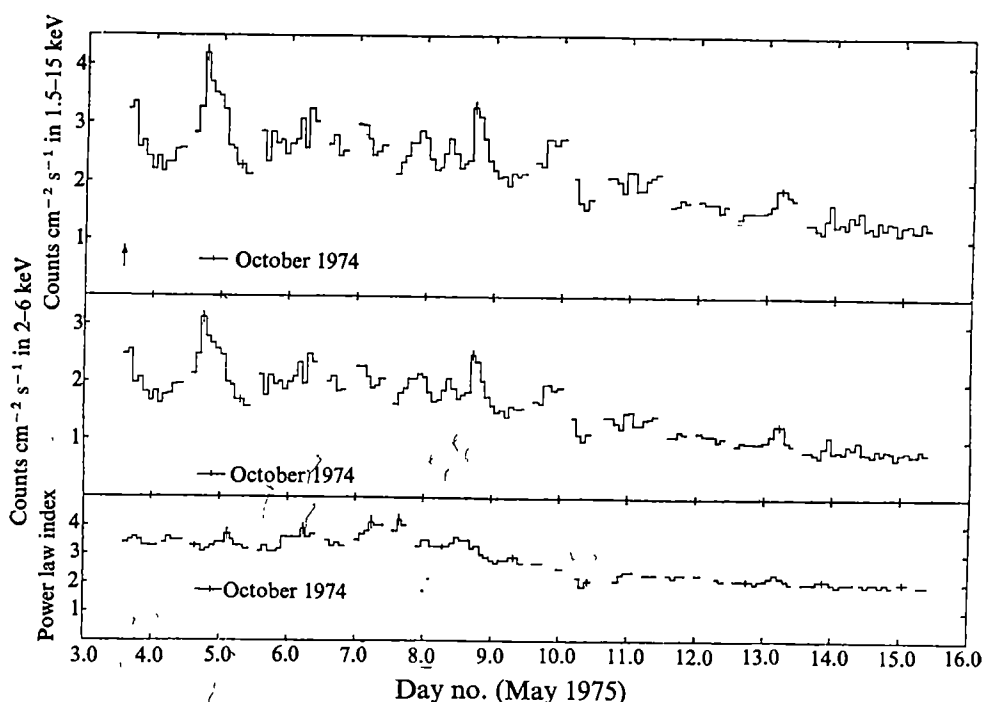
Fig. 1 The X-ray intensity and spectral index for the May 1975 observations with Ariel V.

8 Gorenstein, P., Giacconi, R., and Gursky, H., *Astrophys. J. Lett.*, **150**, L85 (1967).
9 Meekins, J. F., Henry, R. C., Fritz, G., Friedman, H., and Byram, E. T., *Astrophys, J.*, **157**, 197 (1969).
10 Bleach, R. D., Boldt, E. A., Holt, S. S., Schwartz, D. A., and Serlemitsos, P. J., *Astrophys. J.*, **171**, 51 (1972).
11 Cruise, A. M., thesis, Univ. London (1973).
12 Li, F. K., and Clark, G. W., *Astrophys. J. Lett.*, **191**, L27 (1974)
13 Thorne, K. S., and Price, R. M., *Astrophys. J. Lett.*, **195**, L101 (1975).
14 Heise, J., *et al., IAU Circ. no. 2779* (1975).

Fig. 2 Power-law spectra obtained on two representative orbits. *a*, May 6; *b*, May 13. For curve *a* (orbit 3080), $N(E) \propto E^{-3.6 \pm 0.1}$; curve *b* (orbit 3191), $N(E) \propto E^{2.0 \pm 0.05}$. χ^2 per degree of freedom is 4.7 for May 6 and 1.8 for May 13.

a phase of +0.053 using the ephemeris of ref. 2; and in October 1974 a Copernicus observation revealed an event at phase −0.12. The project orbital phases of the events at the time of the 1971 and 1975 transitions are +0.20±0.02 and −0.18±0.01 respectively, which, within the errors, is symmetrical about the line of centres of the binary system.

Without a knowledge of the behaviour of the absorption events following the May 1975 transition, we can only draw attention to this coincidence. But if the absorption events are, as suggested in ref. 2, a manifestation of the gas flow which powers the X-ray source, a correlation between their behaviour and periods of activity is not unreasonable, since changes in the accretion process are a possible trigger mechanism for the transition[13].

This hypothesis does predict that the phase or nature of the absorption events will change significantly following the May 1975 outburst.

P. W. SANFORD
J. C. IVES
S. J. BELL BURNELL
K. O. MASON

Mullard Space Science Laboratory,
University College London,
Holmbury St Mary, Dorking, Surrey, UK

P. MURDIN

Royal Greenwich Observatory,
Herstmonceux Castle,
Hailsham, E. Sussex BN27 1RP, UK

1 Sanford, P. W., Mason, K. O., Hawkins, F. J., Murdin, P. and Savage, A., *Astrophys J. Lett.*, **190**, L55 (1974).
2 Mason, K. O., Hawkins, F. J., Sanford, P. W., Murdin, P., and Savage, A., *Astrophys J. Lett*, **192**, L65 (1974).
3 Gursky, H., *et al., IAU Circ. no. 2778* (1975)
4 Tananbaum, H., Gursky, H., Kellogg, E., Giacconi, R., and Jones, C., *Astrophys. J Lett*, **177**, L15 (1972).
5 Bowyer, S., Byram, E. T., Chubb, T. A., and Friedman, H., *Science*, **147**, 394 (1965).
6 Byram, E. T., Chubb, T. A., and Friedman, H., *Science*, **152**, 66 (1966).
7 Chodil, G., Mark, H., Rodriugues, R., and Swift, C. D., *Astrophys J. Lett*, **151**, L1 (1968)

Another major change in the radio source associated with Cyg X-1

THE sudden appearance[1,2] of a faint radio source inside the probable position region for the X-ray source Cyg X-1 was associated with major changes in the X-ray emission observed[3] during the period February–May 1971. This coincidence established a probable association with the X-ray source. The accurate position of the radio source led to an identification[2,4] with the bright, blue star HDE226868. Subsequent optical investigations have established[5,6] this object to be a binary system with a massive, invisible component that may be a black hole.

Since 1971, no major changes in the Cyg X-1 X-ray and radio source had been reported until the spring of 1975.

We now report further observations of the Cyg X-1 radio source, the most recent of which were made after learning[7] that Cyg X-1 had undergone another major change in X-ray state in, or before, early May 1975. The radio observations were made with the NRAO interferometer at 2,695 and 8,085 MHz and are shown in three parts in Fig. 1. The first part has been published previously[8], but is shown again for completeness and comparison. It shows the 1971 X-ray transition in the 2–6 keV range and the initial appearance of the radio source between March 22 and March 31, 1971, followed by the radio history of the source until October 1972. The second part shows observations between March 1973 and March 1975, which have not been published before. The last part shows observations taken during May 1975 after learning of the recent change in X-ray state[7].

An extensive discussion of the 1971–1972 radio data has been given by Hjellming[8]. The major point of interest is the approximate mean level of 0.015 Jy maintained by the radio source at both 2,695 and 8,085 MHz during the period March 1971 to March 1975. This level is shown by dashed lines in Fig. 1. There are some clear fluctuations about this norm, particularly during extensive observations in October 1972, but a mean level of 0.015 Jy was maintained by the radio source over a time span of four years. The observations of Cyg X-1 at 1,415 MHz by Braes and Miley[2,9] also showed a mean flux level of 0.015 MHz. Thus one of the major characteristics of the Cyg X-1 radio source between March 31, 1971, and March 1975 is a flat radio spectrum, that is, a spectral index of 0.

The Cyg X-1 radio observations on May 9, 1975, are shown on a greatly expanded scale in Fig. 1, because, in addition to the fact that the mean flux density for this day was 0.035 Jy at 8,085 MHz, considerably above the previous norm, the variations from a peak level of 0.045 Jy to a lower level of 0.030 Jy over a period of 5 h on May 9 are real. Subsequent sampling of the radio source between May 10 and May 20 is shown in Fig. 1, plotted on a time scale intermediate between that of the May 9 data and the earlier data.

The mean daily levels of Cyg X-1 during the period May 9–20, 1975, are 0.031 Jy at 8,085 MHz, and 0.002 Jy at 2,695 MHz These levels clearly represent a new state for the radio source. A particularly interesting feature of the new state is the change of the mean spectral index from 0 to 0.31. This means that if the radio emission mechanism is thermal, the source has become slightly optically thick
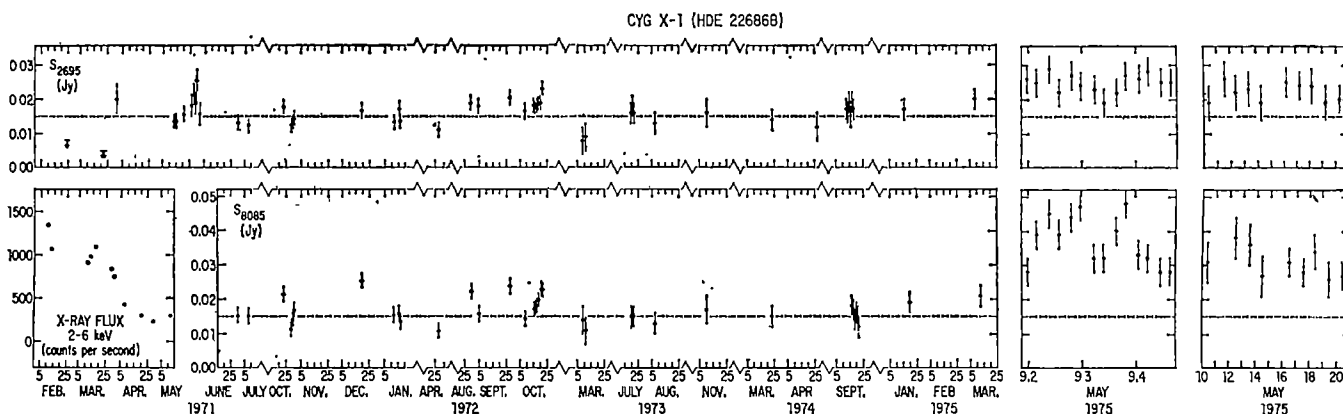
Fig. 1 Radio observations of Cyg X–1 (HDE226868) at 2,695 and 8,085 MHz are plotted as a function of time for the period between February 1971 and May 1975. Also shown is the change in X-ray level in the 2–6 keV energy range during February–April 1971. Note that the time scale for the abscissa is not linear and that different time scales are used in plotting the May 1975 observations.

with an optical depth of 0.17 at 8,085 MHz; however, if the radio emission mechanism is non-thermal, the source is self-absorbed.

The National Radio Astronomy Observatory is operated by Associated Universities, Inc. under contract to the US National Science Foundation.

R. M. HJELLMING
D. M. GIBSON
F. N. OWEN

*National Radio Astronomy Observatory,
Green Bank, West Virginia 24944*

1 Hjellming, R. M., and Wade, C. M., *Astrophys. J. Lett.*, 168, L21 (1971)
2 Braes, L. L. E., and Miley, G. K., *Nature*, 232, 246 (1971).
3 Tananbaum H., Gursky, H., Kellog, E., Giacconi, R., and Jones, C., *Astrophys. J. Lett.*, 177, L5 (1972).
4 Wade, C. M., and Hjellming, R. M., *Nature*, 235, 271 (1972).
5 Webster, B. and Murdin, P., *Nature*, 235, 37 (1972).
6 Bolton, C. T., *Nature*, 235, 271 (1972).
7 Gursky, H., *et al.*, *IAU Circ. no.* 2778 (1975).
8 Hjellming, R. M., *Astrophys. J. Lett.*, 182, L29 (1973).
9 Braes, L. L. E., and Miley, G. K., *Nature phys. Sci.*, 235, 147 (1972).

Can soft γ-ray bursts be emitted by accreting black holes?

THERE are many models for the observed γ-ray bursts[1], but none has proved entirely satisfactory. Here we suggest another possible model: that the γ rays are produced by inverse Compton processes occurring deep in the ergosphere of a fast rotating, accreting, black hole. At the time of burst, plasma, which has an optical depth of the order of the relevant gravitational distances, emerges near the horizon due to an instability, and free falling photons, which are produced by viscous processes in an accretion disk further away, are being scattered off it. In this model the black hole itself is probably a member of a binary system, and, depending on its steady-state accretion rate, may or may not be observable as an X-ray source.

With Katz, we have shown[2] that photons, which free-fall into a Kerr horizon to be Compton scattered by tangentially moving electrons, can acquire very high energies by the Penrose mechanism. The scattered electrons share their negative energy with the protons; thus, the excess photon energy may be supplied mainly by plasma gravitational energy. When the scattered photons escape to infinity, their energies at infinity do not exceed a few $m_e c^2$. We have calculated numerically overall energy spectra for several realistic scenarios of plasmas with

turning points close to the horizon, by the Monte Carlo procedure. Details will be published elsewhere; here we report briefly the emerging qualitative picture.

The maximum energy obtained in the conversion of free-falling X-ray photons into γ rays, increases as the collision point approaches r_{mb}, and can become as high as a few electron masses for $a/M \to 1$. For a canonical black hole[3] ($a/M = 0.998$), and for an X-ray photon of 10 keV falling in radially, energies as great as 600 keV can be reached; with a 50 keV photon falling in radially, energies reach 900 keV. This maximum energy increases when collisions of outgoing photons are considered and, at any rate, increases rapidly as a/M grows above its canonical value.

A small number of collisions help to increase the overall efficiencies by directing additional scattered photons outwards. But when many collisions occur, the photons merely become "thermalised", and the possibility of using free-fall no longer exists. The maximum efficiency was of the order of 10% or higher in the examples considered, and one may also expect a certain anisotropy in the emitted spectra. All our spectra seem to stabilise at spectra similar to those observed[4] when collisions occur deep enough and rotation is fast enough.

The inner disk region is secularly unstable[6,7] and is, therefore, a likely location of matter flow instabilities. The time scale for these instabilities is of the order of a few seconds or less, whereas the time scale for fluctuations around and inside r_{ms} is of the order of a few milliseconds. For accretion rates which are not larger than $10^{-9} M_\odot$ yr^{-1}, and for canonical black holes with masses ~ 1–$10 M_\odot$, the number of (proper) optical depths for high energy photons in the region between r_{ph} and r_{ms} is normally less than one. The suggested γ-ray production mechanism will only be effective for a depth of 1–3 optical paths, which is not an inconceivable number for instabilities in such disks, but is an unlikely number for steady-state flow. This is also in agreement with recent disk structure models (S. L. Shapiro, A. P. Lightman and D. M. Eardly, unpublished and A. P. Lightman, personal communication.)

The initial photons could come from the steady-state flux of the disk or, which seems more likely, from the event itself; one could imagine a sudden burst of matter flow on the companion star[8] to dump a lot of material on the disk, thus producing both a large number of X-ray photons and the necessary deep ergo-sphere plasma flow. The occurrence of softer, and longer lasting, X-ray bursts, on which some of the γ-ray events seem to ride[9], is certainly not inconsistent with such an interpretation. The Vela satellites' γ-ray burst 71–2 (ref. 10), which seems to have come from the general direction of Cyg X-1, occurred close to a remarkable transition in that X-ray source[5]. It is tempting to associate that burst with a disk instability, which is, in turn,

also indicated by the X-ray and radio data (ref. 11 and S. L. Shapiro, A. P. Lightman and D. M. Eardly, unpublished).

We thank J. Katz for discussions.

TSVI PIRAN
JACOB SHAHAM

Racah Institute of Physics,
The Hebrew University, Jerusalem, Israel

1 Ruderman, M., *Proc. seventh Texas Symposium* (in the press).
2 Piran, T., Shaham, J., and Katz, J., *Astrophys. J. Lett.,* **196,** L107–L108 (1975).
3 Thorne, K. S , *Astrophys. J.,* **191,** 597–619 (1974).
4 Cline, T. L., and Desai, U. D., *Astrophys. J. Lett.,* **196,** L43–L46 (1975).
5 Tananbaum, H., *et al., Astrophys. J. Lett ,* **177,** L5–L10 (1972).
6 Pringle, J. E., and Rees, M. J., *Astr. Astrophys.,* **21,** 1–9 (1972).
7 Lightman, A. P., and Eardly, D. M., *Astrophys. J. Lett.,* **187,** L1–L3 (1974).
8 Lamb, D. Q , Lamb, F. K., and Pines, D., *Nature phys. Sci.,* **246,** 52–54 (1973).
9 Palumbo, G. G. C., *et al., Astrophys. J. Lett.,* **189,** L9–L11 (1974).
10 Strong, I. B., and Klebesadel, R. W., *Nature,* **251,** 396–397 (1974).
11 Thorne, K. S., and Price, R. H., *Astrophys. J. Lett,* **195,** L101–L106 (1975).

Mother and baby paradox

THE 'twin paradox' of relativity theory describes the difference in the elapsed time between the twin who stays at home and the one who goes on a space flight and returns. No genuine paradox is involved; elapsed time is not in general independent of the state of motion. In this case the difference in the elapsed time is related to a difference in the (locally measurable) acceleration histories of the twins; the space traveller must suffer an acceleration, however brief, in order to return.

But no paradox is involved, even if there is no locally measurable effect to distinguish the two timekeepers. General relativity provides such possibilities, one of which I describe here.

A mother, desiring her baby to remain young for as long as possible, decided on the following plan of action. Each night, when the baby was asleep, she went out and collected masses from afar, symmetrically, and arranged them in a spherical shell around the baby's crib (herself staying outside and some distance away from the completed shell). The baby's sleep was not disturbed, no acceleration being measurable inside the shell. In the morning before the baby woke up, she dismantled the shell and stored the masses again far away. Doing this carefully, with spherical symmetry, again no acceleration could be felt by the baby. But, in the 12-h period of the night experienced by the mother, the baby had aged only 12 h× $(1-GM/Rc^2)$ (for small values of GM/Rc^2, where M and R refer to the mass and radius of the shell, and G and c are the gravitational constant and speed of light respectively). In the daytime the components of the shell were stored so far apart that the large value of R made the value of GM/Rc^2 negligibly small.

By this procedure the mother's and her baby's timekeeping were caused to be different; yet, unlike the case of the 'twin paradox' of special relativity, without any necessity for a different acceleration history.

T. GOLD

Center for Radiophysics and Space Research,
Space Sciences Building, Cornell University,
Ithaca, New York 14853

Trapped helium and argon and the formation of the atmosphere by degassing

I REPORT here some radiogenic ^{4}He and ^{40}Ar data measured in the glass margins of deep sea pillow basalts, and comment on their applicability to discussions of formation of the Earth's atmosphere by degassing from a solid Earth.

Discussions of such degassing generally assume either catastrophic or continuous degassing (see refs 1, 2 and references therein). In fact, there is no reason why these two sets of models should be considered to be mutually exclusive. The history of the Earth may include both a catastrophic event with an associated 'big burp', and later continuous degassing.

Ozima and Kudo[2] and Ozima[3] have presented calculations which indicate the evolution of the terrestrial ^{40}Ar/^{36}Ar ratio as a function of time, in either catastrophic or continuous degassing models of atmospheric formation. In particular, Ozima[3] has shown that the present ^{40}Ar/^{36}Ar ratio in the mantle is a sensitive indicator of the validity of the catastrophic model. In such models, the degassing is defined by any two of these four quantities: The time of degassing; the fraction of Ar degassed; the K content of the Earth; the present value of the ^{40}Ar/^{36}Ar ratio within the Earth. Ozima showed sets of graphical solutions for these quantities which satisfy the total Ar abundance and the present atmospheric ratio of 295.5.

The rare gases found in deep sea basaltic glasses are clearly those trapped within the rock on its eruption[4-6], due to the high hydrostatic pressures and rapid chilling, and as such represent mantle-ambient gases. A characteristic of those trapped gases is the presence of "excess" radiogenic ^{4}He and ^{40}Ar, in amounts greater than can be accounted for by the decay of U, Th and K subsequent to their eruption[4-7]. Similar "excess" ^{40}Ar contents have been measured in ultramafic intrusives[8] and in continental rock, but their origin is not as clear.

Ozima therefore accepted the ^{40}Ar/^{36}Ar trapped in submarine basalts as representing the isotopic ratio in the upper mantle; the published ratio available to him, ~1,000, could not be reconciled with a catastrophic degassing model of the Earth's atmosphere without the assumption of an unacceptably high K content[3]. He noted, however, following Schwartzman[16], that if the ultramafic values for this ratio (~10,000) were indicative of mantle gases, then the catastrophic model could not be ruled out. The ^{40}Ar/^{36}Ar values shown here in Table 1, measured in the glassy margins of deep sea basalts from both the Atlantic and Pacific Oceans, on both ridge and non-ridge samples, reach values as great as ~15,000. If these values are accepted, Ozima's argument against the catastrophic model must be relinquished. The argument can be replaced, however, with one based on the ^{4}He/^{40}Ar data of Table 1.

Schwartzman predicted a ^{4}He/^{40}Ar ratio in the present-day mantle of between 1.36 and 2.23, depending on the

Table 1 ^{4}He, ^{40}Ar, and ^{36}Ar data in glassy margins of deep sea basalts. The rocks are described in refs 4 and 17. All units are 10^{-8} s.c.c. g^{-1}. The ^{40}Ar reported is total measured ^{40}Ar; correcting for atmospheric contamination does not change the ^{4}He/^{40}Ar results significantly

Sample	^{40}Ar	^{36}Ar	^{4}He	^{40}Ar/^{36}Ar	^{4}He/^{40}Ar
Vema Fracture Zone, Atlantic Ocean					
72	218	0.0175	—	12,500	—
	77	0.024	1,150	3,210	15
	74	0.0167	1,300	4,430	17
88	290	0.055	2,400	5,200	8.3
	330	0.075	2,400	4,400	7.3
92	235	0.025	1,600	9,400	6.8
	204	0.028	2,370	7,300	12
East Pacific Rise, ~10°S					
32	62	0.0174	84	3,600	1.35
	81	0.067	180	1,210	2.2
40	81	0.0104	1,250	7,800	15
	38	0.032	500	1,200	13
44	210	0.0133	1,800	15,800	8.6
	250	0.016	1,970	15,600	7.9
East Pacific Seamount, ~10°S—98°W					
50	157	0.042	2,800	3,740	18
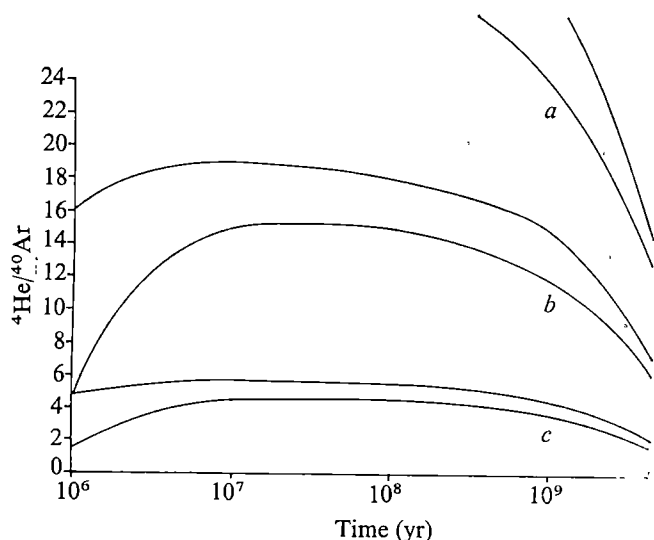
Fig. 1 $^4He/^{40}Ar$ ratio as a function of elapsed time in a closed system, for various values of the K/U and Th/U ratios.

a,   Th/U = 3.6 ⎫
                ⎬ K/U = 1.5×10^3
     Th/U = 2   ⎭
b,   Th/U = 3.6 ⎫
                ⎬ K/U = 3×10^3
     Th/U = 2   ⎭
c,   Th/U = 3.6 ⎫
                ⎬ K/U = 10^4
     Th/U = 2   ⎭

Th/U ratio, for a catastrophic degassing model with very early degassing[1]. He noted that this is in agreement with a ratio estimated for the former environment of lherzolite nodules from Hawaiian nepheline mellilite basalt[9], and pointed out that greater values (2–10) found by Wasserburg *et al.*[10] in volcanic gases might well be due to preferential leaking of ^4He from wall rocks. But some of the values shown here (Table 1) are much too great for such a model, and the preferential leaking argument is much weaker for sub-oceanic mantle sources than for the terrestrial volcanic emanations of Wasserburg *et al.* Since the gas trapping mechanism cannot be 100% efficient, and since in any gas loss ^4He will be lost preferentially to ^{40}Ar, the largest $^4He/^{40}Ar$ ratios reported here are minimum values for the magma-ambient ratios. These values, 15–18, cannot be accounted for at all if the magmatic K/U ratio is similar to the crustal value of 10^4, even less if the ratio is chondritic $(3-6 \times 10^4)$, as seen in Fig 1. Thus the data rule out all models which ascribe the terrestrial atmosphere to any type of degassing of a mantle which is chondritic or crustal with respect to the K/U ratio.

K/U ratios measured on ultramafic rocks lead to an estimated mantle value of $\sim 3 \times 10^3$ (refs 11–14). Assuming this value gives maximum $^4He/^{40}Ar$ values of 15–20 (Fig. 1), but only for an age $\lesssim 10^9$ yr; for a catastrophic degassing model this is the age of the catastrophe, and is much too young The catastrophe is normally identified with formation of the Earth's core, and must have occurred at least within the first 10^9 yr of Earth history; so the age must be $>3.5 \times 10^9$. This necessitates a K/U ratio in the source region $<1.5 \times 10^3$ Such a value is barely within the widest limits set by K/U measurements on ultramafic rocks · 1×10^3–10×10^3 (ref. 12), 1×10^3–5×10^3 (ref. 13). An even smaller K/U ratio would be necessary if core formation (and an associated 'big burp') is to have generated the atmosphere within the first 100 Myr of Earth history, as suggested by Oversby and Ringwood[18].

These data do not suggest that catastrophic core formation, with or without an associated degassing 'burp', could not have occurred. They do suggest that the mantle has not been a closed system with respect to Ar and He

since any such postulated event, and therefore that significant later or continuous degassing from the interior Earth must have occurred The relative contributions to our present atmosphere from a possible early catastrophic event and the necessary later degassing have yet to be worked out.

Further data on $^4He/^{40}Ar$ ratios in more submarine glasses of wider geographic location and of varying magmatic origins, and on K/U ratios in a wider variety of possible mantle materials, should settle the question conclusively.

DAVID E. FISHER

*Division of Marine Geology and Geophysics,
Rosenstiel School of Marine and Atmospheric Science,
University of Miami, Florida*

1 Schwartzman, D W., *Geochim cosmochim Acta*, 37, 2479–2496 (1973).
2 Ozima, M, and Kudo, K, *Nature phys Sci*, 239, 23–24 (1972).
3 Ozima, M., *Nature phys Sci.*, 246, 41–42 (1973)
4 Funkhouser, J. G., Fisher, D. E, and Bonatti, E., *Earth planet. Sci. Lett.*, 5, 95–100 (1968).
5 Noble, C S., and Naughton, J J, *Science*, 162, 265–267 (1968)
6 Dalrymple, G. B., and Moore, J. G., *Science*, 161, 1132–1135 (1968)
7 Fisher, D E., *Earth planet Sci Lett.*, 14, 255–258 (1972)
8 Kirsten, T, and Gentner, W, *Z. Naturforsch*, 21a, 119–126 (1966).
9 Gramlich, J. W, and Naughton, J. J., *J. geophys. Res*, 77, 3032–3042 (1972)
10 Wasserburg, G J, Mazor, E., and Zartman, R E, *Earth Science and Meteoritics*, (edit. by Geiss, J, and Goldberg, E D.), 219–240 (North Holland, 1963)
11 Wakita, H, Nagasawa, H, Uyeda, S., and Kuno, H., *Geochim J*, 1, 183–198 (1967).
12 Fisher, D. E, *Geochim. cosmochim Acta*, 34, 630–634 (1970).
13 Green, D. H., Morgan, J W., and Heier, K S, *Earth planet. Sci Lett.*, 4, 155–166 (1968)
14 Fisher, D E, *Earth Sci Revs* (in the press)
15 Fisher, D E, *Geochim cosmochim. Acta*, 36, 15–39 (1972).
16 Schwartzman, D. W, *Nature phys. Sci*, 245, 20–21 (1973).
17 Fisher, D. E, *Geophys Res. Lett*, 4, 161–164 (1974).
18 Oversby, V. M, and Ringwood, A E, *Nature*, 234, 463–465 (1971)

Geodynamic implications of transverse folding in the Western Alps for the Alpine fold belt

LARGE amplitude translations towards the external parts of the Alpine arc are still considered as the major feature of Alpine tectonics[1-5]. More recent approaches, inspired by concepts of global tectonics[6-8], have led to the development of new models, none of which however accounts for the widespread distribution of early transverse folds which in general are thought to have been formed either before[9] or later [10-13] than the overthrusting, and are considered to be minor structures. New data on the variations in strike of both transverse and longitudinal folds have been gathered over wide areas of the Alps between Savone and the Simplon Pass. The results indicate that in the whole Pennine Zone as well as part of the external zone only two important successive phases of folding have occurred, the trends of which are completely different. Here I am concerned with the 'initial transverse phase' or 'main Alpine phase' of deformation.

The axes of folds generated during the early Alpine deformation, in each of the internal structural zones, now lie roughly in a radial position with respect to the alpine arc (Fig. 1). The S_1 primary cleavage is everywhere related to this phase of deformation The following domains have been distinguished: (1) a zone of incipient slaty cleavage defined by illite-chlorite, for example in the uppermost overthrust sheets of the Briançonnais zone; (2) a zone of flow cleavage parallel to the axial planes of transverse isoclinal folds, for example in the Mesozoic cover of Vanoise[11] or in the Piemontese schistes lustrés of the lawsonite-glaucophane facies[14]; (3) a zone of foliation defined by the preferred orientation of phengite, glaucophane, zoisite, for example in the internal crystalline massifs and Piemontese
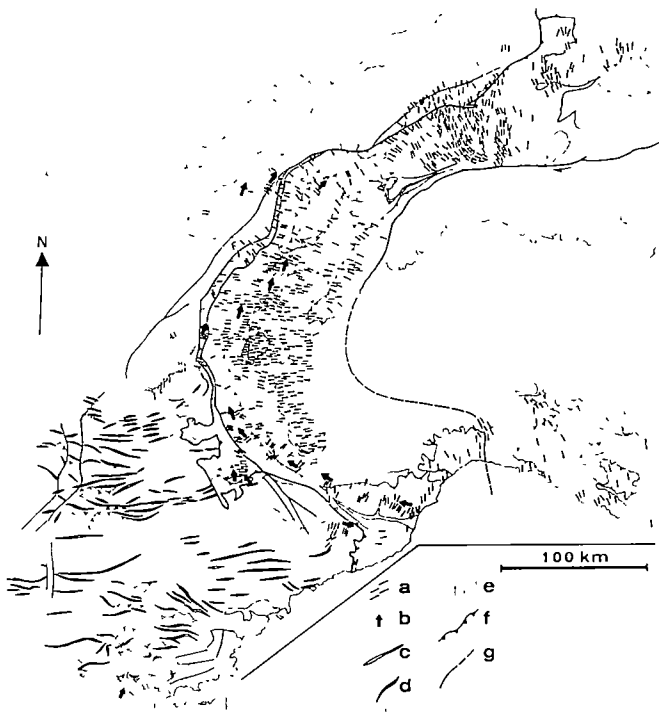
Fig. 1 Trend of transverse folds of the F_1 phase of Alpine deformation (post–Lower Eocene). In the Briançonnais and Grand St Bernard zones after Bertrand[34], Caron, *et al.*[22], Ellenberger[11], Nicolas[14], Vialon[9], Wenk[15], and Wunderlich[36], and personal field data in the Ligurian Alps, Briançonnais, the Aosta Valley and Valais. After Lemoine[37] for the External zone. a, Trend of F_1 transverse folds; b, sense of overturning of F_1 folds; c, pre-Senonian folds of the Devoluy; d, pyreneo-Provencal folds; e, northern limit of Pyreneo-Provencal folds; f, overthrust; g, strike–slip fault.

rocks of the eclogite facies; (4) a zone of foliation defined by biotite, sillimanite and anatectic mobilisates, exposed in the Barrovian metamorphic zones of the central Alps[10] where a later high temperature–low pressure metamorphism is super-imposed[4,15] on an earlier high pressure metamorphism.

The style of the phase 1 folds differs depending on which structural zone they occur in.

Briançonnais cover with both internal and external carboniferous basement. No alpine deformation can be found previous to the phase 1 transverse folds. In many areas, the stratigraphic sequence and sedimentary features in the Lutetian flysch noir allow the sense of overturning of the folds to be clearly established (Fig. 1). On all scales, the transverse folds are similar in style and are syn-metamorphic. They are younger than the nummulitic flysch of lower to middle Eocene age. They were formed during the first large scale tangential movements in the Alps, the amplitude of which still remains unknown, under the

overburden of allochtonous Helminthoid flyschs (Upper Cretaceous age) originally emplaced as olistostromes in the Eocene sea.

Savoy sub-Briançonnais zone. The presence of recumbent to isoclinal refolded folds in this zone has been known for long time[16]. The steeply plunging folds which are overturned to the north were formed also during the first penetrative deformation of post-Eocene age.

Piemontese schistes lustrés. The allochthonous character of the schistes lustrés overlying the internal crystalline basement rocks has been proved on the basis of stratigraphic and palaeontological evidence (substitution de couverture)[11] for the massifs of Vanoise, Ambin[11] and Acceglio[17]. The transverse folds have a similar style and trend in both the schistes lustrés and basement rocks[9,11-13,18]. It is postulated that these syn-metamorphic folds are genetically related to the geosynclinal de nappes[11] or the obduction[19] of Piemontese geosynclinal units of Mesozoic age[20] and remnants of oceanic crust[21]. The underlying heterogeneous basement rocks were subsequently rapidly buried under high pressure–low temperature metamorphic conditions, whereas the overburden required for high pressure–low temperature metamorphic conditions in the Piemontese units was represented by Austroalpine units, such as the Dent Blanche Nappe, or by overthrust sheets of ultra-Piemontese flysch.

In the areas where the S_1 cleavage or foliation is horizontal and unaffected by intense secondary penetrative deformation (Gran Paradiso, Dora Maira, Val d'Aoste), the original trend of the transverse folds has been preserved. But in many areas within the zone of the schistes lustrés, the second phase folds are also isoclinal where formed[14,18,22,23] in calcareous schist units and gliding processes related to the secondary deformation have generated a considerable dispersion of the F_1 folds axes. In contrast, the F_2 slip directions show a more regular pattern[23].

The observations made on the very tight first phase folds which affect the meta-banded cherts of Queyras (Cottian Alps) in which the laminated bedding is still preserved, exclude the existence of an earlier penetrative deformation in the Piemontese zone, as recently suggested[22]. F_1 folds in Piemontese rocks have possibly formed as a result of a long period of penetrative deformation (evidently starting in Upper Cretaceous times as suggested by recent dating of HP minerals[24]) during translations of rock units under conditions of constant stress orientation.

In different metamorphic zones where such relations have been established, the high pressure–low temperature minerals are syn to late kinematic[9-14,16,18,22,23,25,27] with respect to transverse folds. The metamorphic zonation is similar to other orogenic belts with alpine-type metamorphism[28]. The width of each of the three principal metamorphic zones[29]: (1) lawsonite $\pm$ jadeite zone; (2) zoisite–glaucophane eclogite zone; (3) kyanite–eclogite zone, is only compatible with sub-horizontal isograds[30], now refolded in detail. Field evidence, for example west of Gran Paradiso, and between Monte Viso and the Briançonnais units, strongly suggests that the isograds plunge westwards and not eastwards, as recently postulated[28].

Fig. 2 North-south longitudinal combined section perpendicular to F_1 folds, showing the possible configuration of the internal crystalline massifs and the Piemontese obduction zone before the arc curvature. The total thickness of units is compatible with high pressure–low temperature or high pressure–high temperature Alpine metamorphism (after ref. 4).
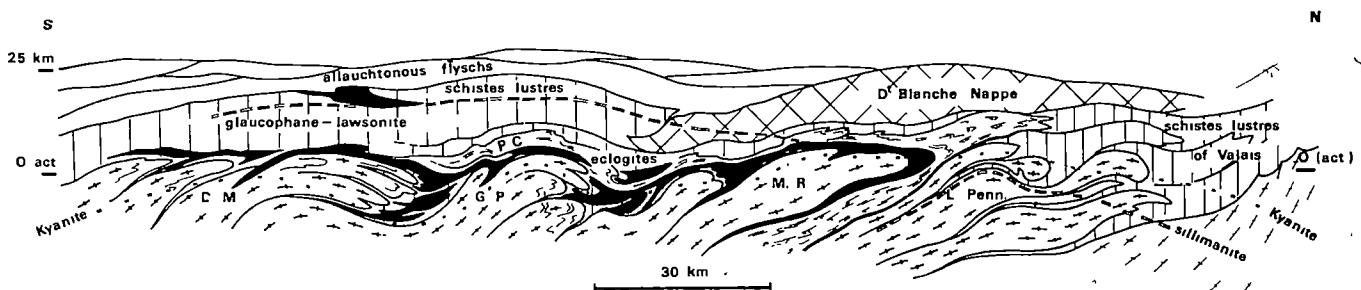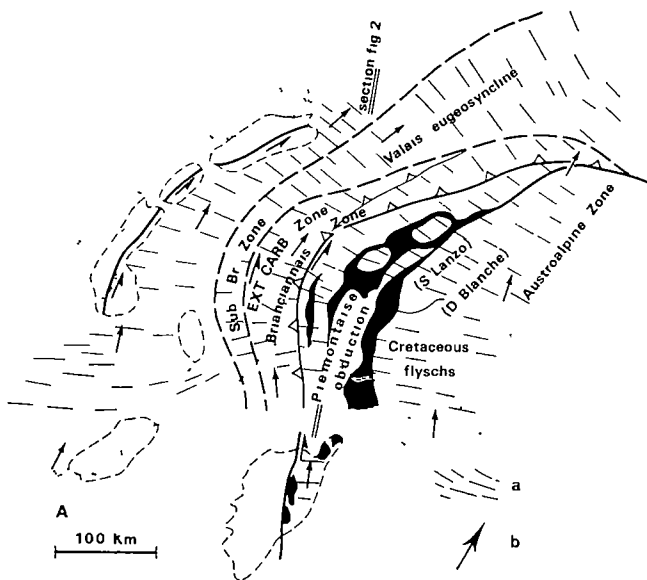
Fig. 3 Possible reconstruction of the western Alps before the arc curvature. The position of the Insubrian plate is the one postulated by Laubscher[7]. Minor rotations of external crystalline massifs are figured. The different structural zones are represented at their actual erosion level. Ophiolites are in black. a, Original trend of F_1 folds; b, direction of tectonic transport.

I would like to put forward a geodynamic model based on this evidence. The radial pattern of transverse folds with respect to the arc is a new factor that has been taken into account in tectogenetic models of the Alps. It seems unlikely that such a pattern could be primary as it would imply a single deformation field of curvature 180°.

In agreement with the data concerning the shortening generated after the F_1 deformation, it seems more likely that the transverse folds were formed when the different structural zones were rectilinear or weakly curved (Figs 2 and 3).

The Dent Blanche Nappe including high pressure granulite facies rocks generally considered to have been formed in the lower crust, belongs to the Insubrian Plate. This plate would have moved initially northwards upon the Piemontese zone, giving rise to high pressure–low temperature metamorphic conditions in the Piemontese units, before its westward later translation. Therefore the basal contact may be regarded as part of a subhorizontal palaeosubduction at the base of the Insubrian sialic crust.

The major thrust, generally interpreted as a retrochevauchement, marking the eastern limit of the Briançonnais zone with external Carboniferous basement, may be regarded as an early major subduction zone, along which the movement revealed by the transverse plunging folds, was essentially horizontal (sinistral wrench fault dipping west). This zone of subduction has been later reactivated and tilted during the F_2 deformation, with a vertical post-metamorphic movement which is particularly spectacular in the Cottian Alps. We recall that the andesitic greywackes of Taveyanne[31] (Priabonian to Lower Oligocene) may be the remnants of a volcanic arc in external position, possibly in connection with westwardly dipping subduction zones. It is not intended here to discuss early kinematics related to downgoing of structural zones and microplates toward the external part of the Alps, which could have acted in the first stages without any penetrative deformation. This subduction was possibly initially related to the expansion of a Tethyan ocean from a Piemontese ridge during Jurassic-Lower Cretaceous times[8].

The constancy of transverse folds in the different structural zones implies horizontal movements and translations of low-angle thrust sheets and small plates of unknown extent towards the NNE, before the arc curvature. At this time (post middle Eocene), the Western Alps must have been a synthetic thrust belt[32]. It is significant that the deformation path compatible with transverse folds is not a contradiction with the motion model in the alpine system at this time deduced from magnetic anomalies in the Atlantic Ocean[33].

The Alpine arc has been generated during the second phase of Alpine deformation, which occurred after a complete change in the relative motion of plates[33]. The arc curvature, formed by the collision of the Insubrian Plate moving westwards (300 km according to Laubscher), reflects the shape of its western edge. The contraction which resulted (Fig. 4), would have been absorbed by three main mechanisms: (1) deep outward under thrusting in the lower crust[35] inducing areas rapidly uplifted and eroded; (2) contraction of each zone by F_2 folding (outward and inward overturned folds are synchronous) mostly responsible for a post-metamorphic flexuration of S_1 cleavage and foliation with sub-vertical Z and for isoclinal folding in the more ductile zones during intense tangential movements. The amount of shortening gradually decreases toward the external zones, where the F_2 folds and cleavage are also younger (post Oligocene) and followed by post Miocene folds, than those of the inner zones (Lower Oligocene); (3) large scale outward translations of the higher units on a continuously rejuvenated and outward moving slope.

This late Alpine phase is characteristic of an antithetic thrust belt[32]. The second phase of Alpine deformation is also responsible for the rapid uplift of high pressure–low

Fig. 4 Transverse schematic section of the western Alps (partly after J. G. Ramsay for the External zone, Ellenberger[11] for the Permo-Carboniferous and Giese[35] for the inferred structure of the Ivrea zone).
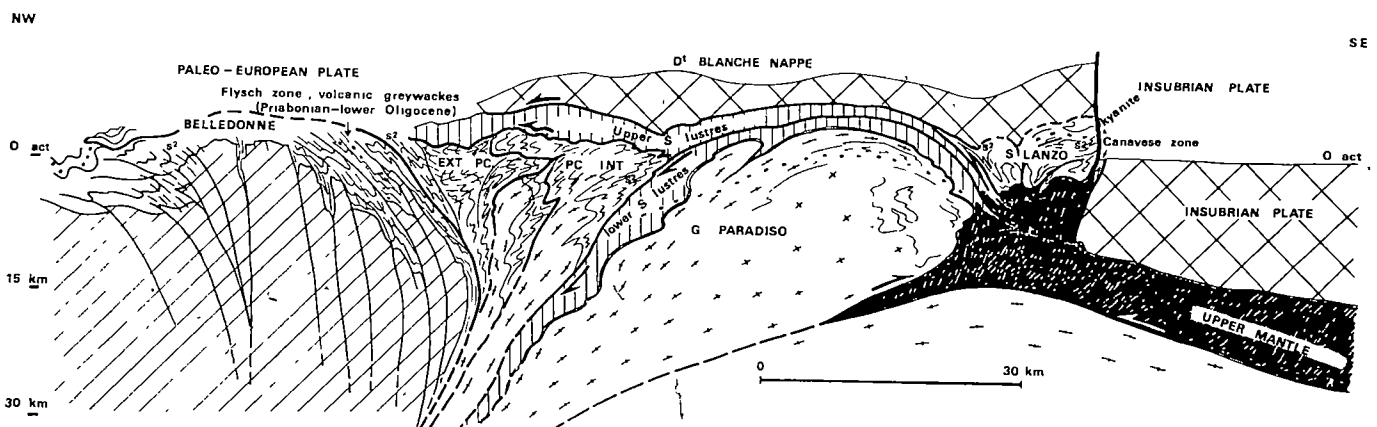
temperature metamorphic zones before the establishment of a normal geothermal gradient[7,28] which would have destroyed the metamorphic assemblages as in the central Alps. A considerable part of the global shortening would also have been absorbed by subduction of the schistes lustrés and internal crystalline basements under the Briançonnais zone and external carboniferous basement.

R. CABY

Centre Géologique et Géophysique,
Université des Sciences et Techniques de Languedoc,
34060 Montpellier Cedex, France

1 Hermann, F., *Carte Géologique au 1/200 000 des Alpes Nord-occidentales* (1938).
2 Staub, R., *Eclog. geol. Helv.*, 44, 30 (1951).
3 Debelmas, J., and Lemoine, M., *Earth Sci. Rev.*, 6, 221 (1970).
4 Niggli, E., *Fortschr. Miner.*, 47, 1 (1970).
5 Trumpy, R , *Eclog. geol. Helv.*, 66, 1 (1973).
6 Bocaletti, M., and Guazzone, G., *Mem Soc. geol. Ital.*, 9, 177 (1970).
7 Laubscher, H. P., *Am. J Sci.*, 271, 193 (1971).
8 Boccaletti, M , Elter, P., and Guazzone, G., *Nature phys. Sci.*, 234, 49–108 (1971).
9 Vialon, P., thesis, Univ. Grenoble (1966).
10 Wenk, E., *Schweiz. miner. petrogr. Mitt.*, 35, 311 (1955).
11 Ellenberger, F., *Mém. expl. Carte géol. Fr.* (1958).
12 Plessmann, W., and Wunderlich, M. G., *Neues Jb. Geol. Paleont. Mh* , 199 (1961).
13 Chatterjee, N. C., *Geol. Rdsch.*, 53, 536 (1963).
14 Nicolas, A., *Schweiz. miner. petrogr. Mitt.*, 49, 360 (1969)
15 Wenk, E , *Fortschr. Miner.*, 47, 34 (1970).
16 Barbier, R , *Mém. expl Carte géol. Fr.* (1948).
17 Lemoine, M., *C.r. somm. Séanc. Soc. géol. Fr*, 9, 234 (1960).
18 Caby, R., *Geol. alpine*, 44, 95 (1968).
19 Coleman, R. G., *J. geophys. Res.*, 76, 1212 (1971).
20 Lemoine, M., *Geol alpine*, 47, 181 (1971).
21 Nicolas, A., and Jackson, X., *Schweiz miner. petrogr Mitt.*, 53, 3, 479 (1971).
22 Caron, J. M., Schumacher, F., and Tricart, P., *Sci. Geol.*, *Strasbourg*, 25, 2–3 (1973).
23 Caron, J. M., *Geol. alpine*, 50, 45 (1973).
24 Hunziker, J. C., *Annls Soc. géol. Belg.*, 94, 116 (1971).
25 Gay, M., *Archs Sci.*, Geneve, 25, 1 (1972).
26 Chadwick, B., *Bull. geol. Soc. Am.*, 79, 1123 (1968).
27 Dalpiaz, G V., *R. Soc. ital. miner. petrol* , 27, 437 (1971).
28 Ernst, W. G., *Contr. Miner. Petrol.*, 34, 43 (1971)
29 Kienast, J. R., and Velde, B., *C.r. hebd Séanc. Acad. Sci. Paris D*, 271, 637 (1970).
30 Saltot, P., *C.r. hebd Séanc. Acad. Sci. Paris* (in the press).
31 Martini, J., and Vuagnat, M., *Geol. Rdsch.*, 57, 264 (1967)
32 Roeder, D. H., *J. geophys. Res.*, 78, 23, 5005 (1973).
33 Dewey, J. F., Pitman, W. C., Ryan, W. B. F , and Bonin, J., *Bull. geol. Soc. Am.*, 84, 3137 (1973)
34 Bertrand, J. M., *Geol. Alpine*, 44, 55 (1968)
35 Giese, P., *Schweiz. miner. petrogr. Mitt* , 48, 1, 261 (1968).
36 Wunderlich, H. G., *Geol. en Mijnbow*, 42, 155 (1963)
37 Lemoine, M., *Geol. Rdsch.*, 61/3, 975 (1972).

Triassic seaways and the Jurassic Tethys Ocean in the central Mediterranean area

IN the central Mediterranean area phases of middle Triassic and middle Liassic rifting are recognisable. The former produced only relatively deep pelagic basins founded on thinned continental crust; the latter, however, marked the opening of the central Tethys. The Porphyrit-Hornstein and the Diabas-Hornstein formations[1] are the results of such phenomena. During Dogger and Malm times the spreading continued and a large amount of oceanic crust was formed. The Tethys Ocean opened parallel to the long axes of some Triassic deep sea basins and across the long axes of others. Consequently, the positions of Triassic seaways along the southern continental margin of the Tethys 'geosyncline' are defined by the effects of at least two main palaeotectonic events.

In the Triassic System the Germano-Andalusian facies is characterised by prevalent continental deposits, whereas the Alpine facies is characterised by marine deposits. In the circum-Mediterranean area the basements of both Germano-Andalusian and Alpine facies sediments everywhere comprise continental crust which may underlie Palaeozoic sediments and volcanics. Only in the Antalya Nappe (Turkey) are there indications that a crust of oceanic composition underlies the Alpine facies sediments.

The Alpine facies, occurring mainly in the Upper Triassic, is generally represented by shallow water carbonates (dolomites and limestones), and in certain areas by evaporites. Locally,

Halobia limestones are known, showing relatively deep sea environments. The outcrops of *Halobia* limestones form narrow belts, more or less disconnected, which stretch along the circum-Adriatic chains from Greece to Sicily.

The complete Triassic sequence containing the *Halobia* limestones is not much varied in the whole Mediterranean region. The Lower Triassic is represented by shallow water, terrigenous sediments and acid volcanics, which are a prolongation in time of the Upper Permian facies. The Middle Triassic is represented by terrigenous sediments simulating flysch deposits (Monte Facito Formation in the Apennines and Sicily; Montenegro 'flysch' in Yugoslavia), by mafic volcanics (Porphyrit-Hornstein Formation in Yugoslavia and in Greece), and less frequently by nodular limestones like Ammonitico Rossa (Han Bulog Beds in Yugoslavia and in Greece). The Upper Triassic is represented by *Halobia* limestones with interbedded rare mafic lavas and hyaloclastites, and locally by nodular limestones like Ammonitico Rosso (Hallstatt Beds in the eastern Alps).

The *Halobia* limestones have been considered as markers of an oceanic Palaeo-Tethys in the central Mediterranean area[2,3]. I suggest, however, the basins to which pelagic pelecypods and ammonites migrated originated from an eastern oceanic Palaeo-Tethys and penetrated deeply into a continental Palaeo-Mediterranean gulf as 'seaways' founded on thinned continental crust. Among these Triassic seaways the most considerable is the Pindos Basin, which stretches for more than 1,200 km through Greece, Albania and Yugoslavia. On the opposite side of the Adriatic Sea, after a gap of some 100 km, the basin is recognisable in the southern Apennines–Sicily arc as unconnected outcrops stretching for about 800 km.

In Jurassic times a real ocean separated the European and the African continents. Contemporaneously, large shearing movements began between Europe and Africa as a result of the opening of the southern Atlantic[4].

Jurassic Tethyan deposits consist of mafic and ultramafic rocks, radiolarites, pelagic limestones, claystones and sandstones, frequently affected by high pressure–low temperature metamorphism.

A basic assumption in any attempt at a palinspastic restoration of Triassic and Jurassic palaeogeography[3,5] is that the volume of continental crust remained roughly the same before and after the Alpine deformation. Consequently, the kinematic system is considered a closed system. But a lot of continental crust must have disappeared into the asthenosphere[6,7] and, therefore, huge volumes (or surfaces in a two-dimensional

Fig. 1 Palinspastic restoration of Upper Triassic palaeogeography in the central Mediterranean area. *a*, Stable areas with slight subsidence; *b*, subsided basins of Germano-Andalusian facies; *c*, shallow-water carbonates of Alpine facies; *d*, 'seaways'; 1, Sicani zone; 2, Imerese zone; 3, Lagonegro zone; 4, Triassic pelagic sediments of Slovenia; 5, Budva-Kotor zone; 6, Pindos zone; 7, Australpine of Hallstatt; 8, Australpine of the Gemerides; 9, Australpine of the Balaton Lake; 10, Subpelagonian Triassic pelagic limestones of Serbia; 11, Subpelagonian Triassic pelagic limestones of Northern Pindos and Othrys.
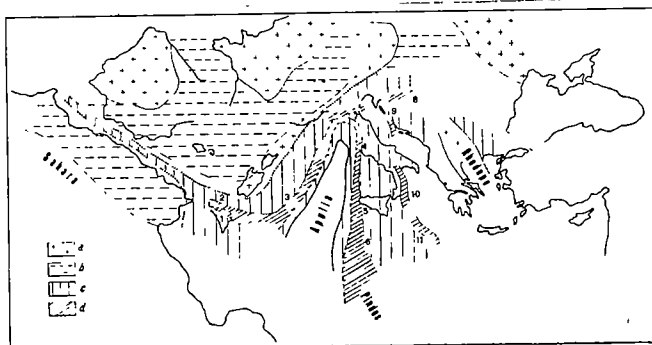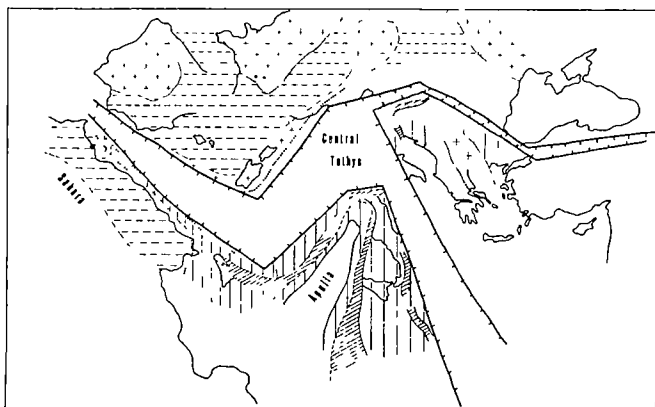
Fig. 2   The Tethys Ocean in the central Mediterranean area.

calculation) have been taken out of the system. These lost volumes are not counterbalanced by additional small volumes of new oceanic crust. In any palinspastic restoration it is therefore necessary to smooth out all the nappes of the peri-Mediterranean Alpine systems to calculate the real shortening of the plate margins during the Alpine compression. Figures 1 and 2 attempt such a palinspastic restoration for the Upper Triassic and Upper Jurassic. The original position of the palaeogeographical units has been obtained by fixing the 'stable' European and the Saharan plates, smoothing out folds and nappes, removing the main lateral displacements and connecting those belts which are common to the African and European margins. This construction of the Western part of Fig. 2 relied upon the palinspastic restoration of Laubscher[7].

This reconstruction produces an area corresponding to the Alpine facies, forming a gulf open in the direction of the Palaeo-Tethys and surrounded by regions characterised by Germano–Andalusian facies. In these regions it is possible to distinguish stable zones, slightly subsident and unaffected by synsedimentary tectonics, and less stable or clearly unstable zones, characterised by high rates of subsidence and sedimentation, synsedimentary faulting and mafic volcanism.

In the area comprising Alpine facies the palaeogeography changes radically from the Lower to the Upper Triassic because of a Middle Triassic tectonic phase, which was responsible for the tectonic regime immediately before the opening of the Tethys. This tectonic phase included an attempt at rifting, which produced a complex system of tensional faults that crossed the Palaeo-Mediterranean Gulf in several directions. The faulted blocks underwent different vertical movements. The uplifted blocks often underwent severe erosion, whereas flysch-like sediments were deposited on the downshifted blocks. Contemporaneously, widespread mafic magmatic activity (comprising an alkali-basaltic series) occurred along the main faults. Deep sea basins developed along preferential directions (probably corresponding to strips of crustal thinning) within the Palaeo-Mediterranean Gulf, allowing the diffusion of the pelagic fauna from the Triassic Palaeo-Tethys through Turkey, Greece, Albania, Yugoslavia, Hungary, Czechoslovakia, Austria, Italy, and as far as western Sicily. The absolute homogeneity of the fauna proves that close intercommunications were established between all of the seaways. Figure 1 shows the region during the late Triassic.

During Liassic times, starting mainly in the Middle Liassic, important new events occurred in the central Mediterranean area: along giant tensional fractures, partly parallel to, and partly discordant with, the former seaways, the Tethys Ocean began to open. In the internal nappes of the Dinarides and of the Hellenides at least two main ophiolite assemblages deriving from the oceanic Tethys may be distinguished: the Diabas-Hornstein Formation, and the ultrabasic massifs at Zlatibor and Vourinos, with their basalts and sediments. The

Diabas-Hornstein Formation comprises alternated diabases, radiolarian cherts, sandstones, claystones, graded calcareous microbreccias, allodapic and pelagic limestones, and intraformational conglomerates. This sequence may overlie Jurassic limestones founded on continental crust[8].

The generally tectonic basal contact and the occurrence of slices of serpentinites between the Jurassic limestones and the Diabas-Hornstein Formation suggest that the latter (at least in part) was possibly founded on oceanic crust. On the other hand, the occurrence of terrigenous material and of neritic calcareous turbidites suggests provenance on a very near continental platform. I suggest that the Diabas-Hornstein Formation is the product of the Liassic rifting which marked the beginning of the opening of the Tethys Ocean, so it may be considered as a marker of the continental margin or the continental rise. As the opening of the Tethys Ocean continued, the areas in which the Diabas-Hornstein Formation had been deposited were shifted laterally and, according to plate accretion models, new areas of oceanic crust were generated in their place. These new areas of oceanic ridges which are, of course, younger than the Diabas-Hornstein Formation, are today recognisable as the ultramafic massifs.

In the Alps and in the Apennines this distinction between initial rifting (Diabas-Hornstein Formation) and real ocean spreading (ultramafic massifs) of the central Tethys is not so clear as it is in the Dinarides, but it is probable that the Ligurian and Piemontese ultramafic rocks represent an ancient, oceanic ridge area, whereas parts of the Southpennine *Bundnerschiefer* represent an equivalent of the Diabas-Hornstein Formation.

During the Middle and Upper Liassic the combination between the Atlantic and the Tethyan movements produced a renewal of synsedimentary tectonic activity, and consequently the palaeogeography became modified not only around the central Tethys, but also around the former Triassic seaways. For example, in western Sicily the former Imerese seaway, which during the Upper Triassic ended at the longitude of Palermo, suddenly lengthened westwards during the Liassic, in the direction of the Maghreb along a newly generated deep furrow in which radiolarian oozes and calcareous turbidites stratigraphically overlie collapsed shallow water dolomites[9]. Contemporaneously, in areas of maximal tension (Imerese, Sicani, Pindos), flows of alkali-basalts were erupted.

During post-Jurassic times the Triassic seaways near to the margin of the Tethys Ocean, and those which were cut across by the opening of the ocean, were affected by Cretaceous compression. These orogenic phases, the earliest in the central Mediterranean area, were responsible for the reduction, and the local disappearance, of large oceanic zones. In Greece (Northern Pindos[10] and Othrys), sediments deposited very near to the Tethys Ocean–Triassic seaways were affected by Cretaceous compression, and were involved in the building of the ophiolite nappes.

On the other hand, the continuing sedimentary history of those seaways which lie more within continental areas, will cease only after the collision between Europe and Africa. In Sicily (Sicani Mountains[11]), such sedimentary deposits were affected by compression during the Upper Miocene, when Europe and Africa had already collided and a lot of continental crust from both plates had disappeared because of subduction.

P. SCANDONE

*Istituto di Geologia e Geofisica,
Università degli Studi di Napoli,
Naples, Italy*

1 Ciric, B., and Karamata, S., *Bull. Soc. géol. Fr*, 2, 276–380 (1960)
2 Wezel, F C, *Riv Miner Sicily*, 21, 187–198 (1970)
3 Dewey, J. F., Pitman, W C, Rian, W. B. F, and Bonnin, J, *Bull. geol. Soc. Am.*, 84, 3137–3180 (1973).

4 Pitman, W. C , and Talwani, M., *Bull. geol. Soc. Am.*, **83**, 619–646 (1972)
5 Bosellini, A , and Hsu, K. J , *Nature*, **244**, 144–146 (1974).
6 Laubscher, H. P , *Am. J Sci.*, **271**, 193–226 (1971).
7 Laubscher, H. P , *Le Scienze*, **72**, 48–59 (1974).
8 Auboun, J., *Bull. Soc géol. Fr* , **15**, 5–6, 425–462 (1974)
9 Giunta, G., and Liguori, V , *Boll Soc. geol ital.*, **92**, 903–924 (1973).
10 Terry, J , *C. r. Seanc. Soc. géol Fr.*, 384–385 (1971).
11 Scandone, P., Giunta, G., and Liguori, V., *C.I E.S M.XXVI Congr.*, Monaco, 1974.

Laser microprobe mass analysis of organic materials

LASER microprobes currently in use combine a laser microscope with a light emission spectrometer[1-3]. Recent improvements in instrumentation have demonstrated the limitations of this method with respect to spatial resolution (some μm) and detection capability (10^{-13}–10^{-15} g). Much lower detection limits (10^{-18} g) have been obtained in ultrathin sections with electron probe X-ray microanalysers[4,5], although the latter, however, do not satisfy many needs of biologists. Neither trace
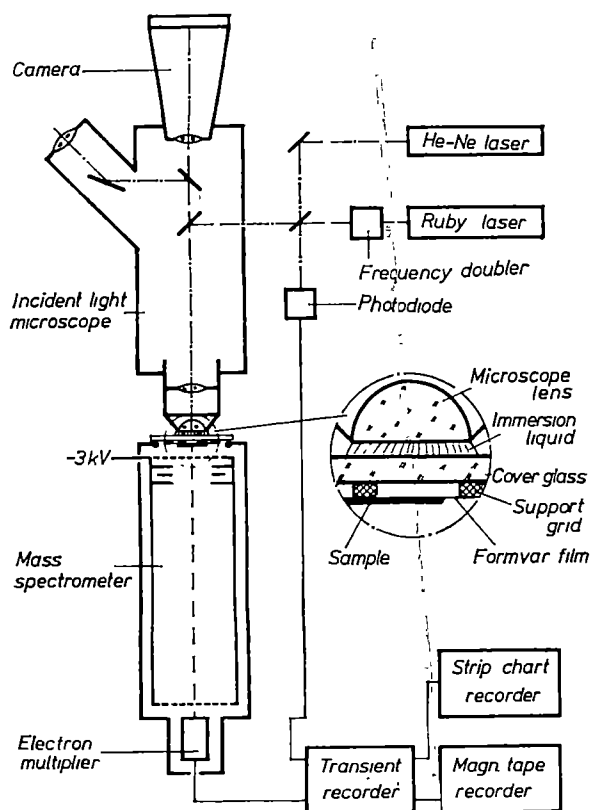
Fig. 1 Schematic diagram of laser microprobe mass analyser.

element detection in the parts per 10^9 range nor analytical information about organic constituents car be obtained. In the case of the main physiological electrolytes, biologists are primarily interested in the cellular and subcellular transport

kinetics of Na^+, K^+, Mg^{2+}, and Ca^{2+}, indicating a need for microprobe techniques capable of analysing isotopes. A mass spectrometer in combination with a laser microprobe seems to be a very attractive approach. In principle, it should enable not only high sensitivity but also isotope analysis and the 'fingerprinting' of organic molecules, provided they do not decompose into fragments too small for recognition of the parent molecules. The special requirements for biomedical application are satisfied by the laser microprobe mass analyser (LAMMA) described here.

The instrument (Fig. 1) combines a time-of-flight mass spectrometer with a laser optical device. The latter consists of an incident light microscope and a Q-switched, frequency-doubled ruby laser ($\lambda = 347$ nm). The specimen is mounted on a form-var-coated electron-microscope grid fixed to the lower side of a microscopic cover glass with no further support underneath (Fig. 1). In addition the cover glass serves as the vacuum window of the time-of-flight mass spectrometer. This arrangement enables free expansion of the laser-generated microplasma into the mass spectrometer. Maximum optical resolution is obtained using an immersion objective. Sampling areas (down to 0.5 μm in diameter and almost limited by diffraction) could be achieved reproducibly in a rather broad variety of unstained biological materials by adjusting the laser power density in the focus between 5×10^8 and 5×10^9 W cm^{-2} (ref. 6).

The time-of-flight spectrometer consists of a 3 kV acceleration grid, 4 mm below the sample plane, an einzel lens, a field-free drift tube of approximately 1 m in length and an electron multiplier as ion detector. The overall transmission of the mass spectrometer depends on the directional distribution of the initial ion velocity. Rough calculations indicated that a transmission of 1 % or better could be achieved if the initial energy of the ions remained below 50 eV. Measurements showed that, in the conditions chosen, ions had mean initial energies of 5–30 eV (ref. 6). Accordingly the calculated peak half width at mass number 39 is less than 0.15 a.m.u. which means that, theoretically, a resolution of $m/\Delta m = 260$ should be possible. The experimental peak half-width is, however, limited by the recording electronics, which consisted of an impedance matching preamplifier, a fast transient recorder and a strip chart or magnetic tape recorder. To determine sensitivity and resolution of the microanalyser as well as the reproducibility of recorded spectra, standard specimens were prepared according to a suggestion made by Spurr[7]. Organometallic complexes formed from macrocyclic polyethers and inorganic ions such as Li^+, Na^+, Mg^{2+}, K^+, Ca^{2+} and Co^{2+} were dissolved in epoxy resin to give final doping concentrations in the range 10^{-2}–10^{-6} weight fraction. Sections (0.1–1.0 μm thick) were cut on a microtome.

Figure 2 shows two mass spectra obtained from such epoxy standards doped with Li, Mg, and Co. In spectrum (a) the expected signals of Li, Mg, and Co can be readily identified as well as those from Na and K contained in the epoxy resin as impurities. Most of the other peaks seem to represent fragments of the organic matrix. In spectrum (b) many of these signals exceed the dynamic range of the transient recorder, which was set to obtain optimum recording of the
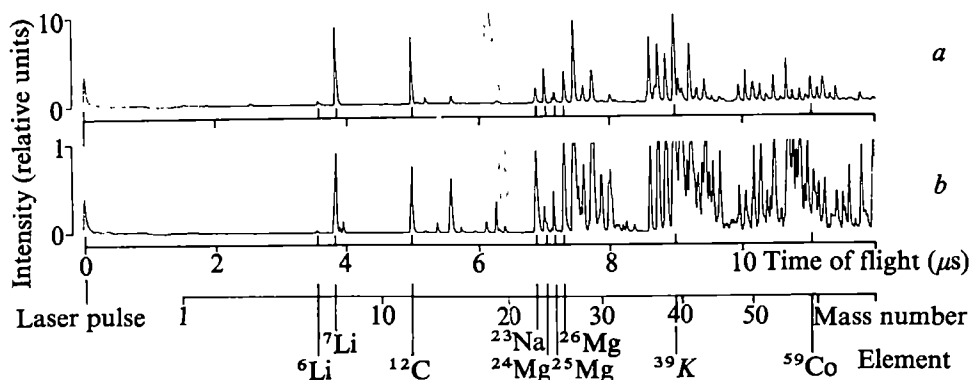
Fig. 2 Mass spectra obtained from epoxy resin, doped with L, Mg, and Co. a, Sample doped with 4×10^{-5} weight fraction (40 p p m.) of each of the inorganic ions. b, Sample with a doping of only 5×10^{-6} (5 p.p.m). b was recorded at an instrument sensitivity ten times that of a. In both cases the sampled volume was 3.5×10^{-13} cm^3 corresponding to a mass of 3.9×10^{-13} g (diameter 1.5 μm, thickness 0.2 μm). The electronically added laser signal serves as zero time mark of the time-of-flight scale and as laser output control.

Li isotopes. The relative peak heights of [6]Li and [7]Li correspond quite reproducibly with the natural abundances of 7.5% [6]Li and 92.5% [7]Li. For Mg this correspondence is lost in the statistical fluctuations of the rather small signals at mass numbers 24 (natural abundance 79%), 25 (10%) and 26 (11%). A partial masking of the Mg signals by mass interfering molecules may be one of the reasons for this.

The overall sensitivity of the instrument is estimated from spectrum (b). With a probed mass of 3.9×10^{-13} g and [6]Li present at a weight fraction of 4×10^{-7} (0.4 p.p.m.), 1.4×10^{-19} g or 1.4×10^{-4} atoms [6]Li were detected. This sensitivity is at least one order of magnitude better than that achieved in electron probe X-ray microanalysis. Further improvements are possible as in this experiment neither the multiplier nor the preamplifier were optimised.

Peak half-widths so far obtained are less than 0.3 a.m.u. at mass number 39 (K). This is, of course, more than is needed to resolve natural isotopes. In mass ranges in which large amounts of organic fragments occur, however, it may be difficult to separate small peaks of trace elements from those of components with nominally the same mass number, for example, Si may be obscured by C_2H_4 or CO. On the other hand, preliminary spectra from freeze-dried specimens of kidney, frog skin, or human erythrocytes have shown Na and K peaks much in excess of those of the organic fragments, the ion yield of which is obviously comparatively small.

In spite of the positive features of the method with respect to sensitivity and resolving power, quantitative analysis still poses some problems. Both ion yield and transmission depend on the ion species under investigation. Moreover the laser radiation–solid interaction involves some highly non-linear processes which make reproducibility from shot to shot rather poor. The best results so far achieved with standard specimens exhibit a s.d. of ±20% in the case of [7]Li. The signals of other elements vary by more than an order of magnitude (C peaks, Fig. 2). This is approximately twice the s.d. found in electron probe X-ray microanalysis of similar specimens[8]. It is expected that systematic optimisation of the laser operating conditions will overcome this problem.

FRANZ HILLENKAMP
EBERHARD UNSÖLD

Gesellschaft für Strahlen-
und Umweltforschung mbH München,
D-8042 Neuherberg, Federal Republic of Germany

RAIMUND KAUFMANN
RAINER NITSCHE

Universität Düsseldorf,
Institut für klinische Physiologie,
D-4000 Düsseldorf, Federal Republic of Germany

[1] Glick, D., in *Modern Techniques in Physiological Sciences* (edit. by Gros, J F, *et al*), 217–223 (Academic, London and New York, 1973).
[2] Marich, K. W, in *Microprobe Analysis as Applied to Cells and Tissues* (edit. by Hall, T. A., *et al*), 15–31 (Academic, London and New York, 1974)
[3] Moenke, H. and Moenke, L, *Laser Micro-Spectrochemical Analysis* (Hilger, London, 1973).
[4] Hall, T. A., in *Physical Techniques in Biological Research*, 1A, (edit by Oster, G., *et al.*), 157–275 (Academic, London and New York, 1971).
[5] Hall, T. A., in *Microprobe Analysis as Applied to Cells and Tissues* (edit by Hall, T A., *et al.*), XV–XIX (Academic, London and New York, 1974)
[6] Hillenkamp, F., Kaufmann, R., Nitsche, R , Remy, E., and Unsold, E., *ibid.*, 1–14
[7] Spurr, A. R , *ibid*, 213–227.
[8] Dorge, A., Gehring, K., Nagel, W., and Thurau, K., *ibid.*, 337–349.

Stability of complex ecosystems

ECOLOGISTS have generally held that a complex ecosystem is more likely to be stable than is a simpler one. Gardner and Ashby[1] have shown, however, that for randomly connected linear systems the probability that the equilibrium will be stable actually decreases as the complexity increases. Of course, whether or not this is a contradiction depends very much on what is meant by stability. It is doubtful that field ecologists are really thinking of the relative likelihoods that systems of

different types will persist; it is, after all, only those systems which persist which are observed and about which generalisations are made. The reason for connecting stability and complexity seems rather to have been the observation that large scale population oscillations are much more common in simple ecosystems than in complex ones[2,3]. We have therefore considered the following problem: Given that a system is near a stable steady state, will increased complexity tend to increase or to decrease the rate at which perturbations die out?

We have constructed random matrices according to the procedure suggested by Daniels and Mackay[4]: the diagonal elements a_{ii} ($i=1, 2, \ldots, N$) were taken from the uniform distribution on $(-1, -0.1)$ and a specified fraction C of the off-diagonal elements were taken from the uniform distribution on $(-1, 1)$. The remaining elements were set equal to zero. We found the eigenvalues λ_j, discarded all those matrices which were not stable, and for the stable matrices computed the value of

$$D = \frac{1}{N} \sum_{j=1}^{N} \exp\{\mathrm{Re}(\lambda_j)\}$$

This represents the magnitude after one unit of time of a unit perturbation which was originally distributed equally in all the eigendirections, and is thus a measure of the mean damping in the associated linear system. We also computed D averaging over all matrices, stable or not, but, as we expected, D then increased with both N and C: for $N=8$ and $C=0.6$, for example, D was 0.721.

The observed lack of dependence of D on complexity may be surprising, but there is evidence to show that it is not artefactual. For example, the similar expression $\exp(\Sigma \mathrm{Re}(\lambda_j)/N)$, which is formed from the trace of the stability matrix, is totally independent of both N and C, and although it is not in general equal to D, the difference is much smaller when all the $\mathrm{Re}(\lambda_j)$ are negative, as the effect of exponentiation is less. Consider also the two-species linear system, the eigenvalues for which are

$$\lambda = \tfrac{1}{2}[a_{11}+a_{22} \pm ((a_{11}-a_{22})^2 - 4a_{12}a_{21})^{1/2}]$$

If the eigenvalues are complex, that is, if there are any oscillations at all, then the rate of damping is entirely independent of the interaction terms a_{12} and a_{21}. If, on the other hand, the eigenvalues are real, then if the system is to be stable the eigenvalues are restricted to the range $(a_{11}+a_{22}, 0)$. If a_{12} and a_{21} are larger than a_{11} and a_{22}, then the effect will not be to increase (or decrease) the damping, but either to produce oscillations or destabilise the system, depending on the sign of $a_{12}a_{21}$. These features are consistent with those found numerically in larger systems.

We conclude that whereas increasing N or C decreases the probability that a randomly connected linear system will be stable, once it is given that such a system is stable, the rate at which perturbations die out does not depend on the complexity. Damping seems to be caused mainly by the self-limitation terms, whereas the chief effect of interactions between species is to produce oscillations.

How can we reconcile these results with the existence in nature of complex ecosystems which are stable and in which there do not seem to be population oscillations on the same scale found in simpler systems? May[5] has argued that we need not be unduly concerned about the prediction that complex systems are likely to be unstable, because what we observe is not a random sample of arbitrary systems but rather only those which have evolved in such a way as to produce stability and therefore persistence. But this line of reasoning does not explain the lack of oscillations in complex systems. We believe that the solution to both problems may lie in a difficulty in the definition of complexity first pointed out by Somoraji and Goswami[6]. The stability matrix is a matrix of first derivatives, and C' (which ought properly to be called the linear connectivity) thus measures not the total number of inter-

Table 1   Persistence of oscillations as a function of complexity

N	C=0 σ	C=0.3 μ	σ	n	C=0.6 μ	σ	n	C=0.9 μ	σ	n
4	0.08	0 594	0 08	437	0.596	0.08	302	0.596	0 07	217
5	0.07	0 590	0.07	391	0.606	0.06	240	0.596	0.07	134
6	0 06	0.589	0 06	354	0.600	0.06	138	0.608	0 05	70
7	0.06	0.594	0.06	292	0.600	0 05	83	0 616	0 05	35
8	0 05	0.594	0.06	194	0.596	0.05	46	0.593	0.04	19
9	0 05	0 598	0 05	149	0.601	0.05	27	0.596	0 04	6
10	0.05	0.585	0 04	85	0.621	0.03	9	—	—	0
11	0.05	0 603	0 03	56	0 569	0.01	2	—	—	0
12	0.04	0.599	0.04	27	—	—	0	—	—	0

Mean (μ) and s.d. (σ) of D for values of N and C chosen to cover the range for which the probability of stability is non-negligible. Number of stable matrices (n) out of 500 in each case is also shown When $C=0$ then $\mu=0 597$ for any N.

actions between species, but only those such that at equilibrium a small change in the population density of one species produces a direct change in the net specific growth rate of another. There seems no reason to suppose that the majority of interactions within an ecosystem fall into this class; it excludes, for example, the obviously vital connection between a predator and its only prey, in any situation in which the prey is in excess and the predator is limited by some other factor, such as space. If large, complex systems have the property that their linear connectivity is low, then they are more likely to be stable, and if stable, are more likely to be over-damped than to oscillate. An example of this behaviour can be found in the analysis of an n-species food chain[7]. And only ecosystems with relatively few species can have a sufficiently high linear connectivity to produce oscillations without becoming unstable— which would explain why it is that population oscillations are observed in the arctic but not in the tropics.

This work was supported in part by NERC.

P. T. SAUNDERS
M. J. BAZIN

Departments of Mathematics and Microbiology,
Queen Elizabeth College,
London W8 7AH, UK

1 Gardner, M R., and Ashby, W R , *Nature*, **228**, 784 (1970).
2 Elton, C A , *Ecology of Invasions by Animals and Plants* (Methuen, London, 1958)
3 Odum, E P , *Fundamentals of Ecology* (Saunders, Philadelphia, 1953)
4 Daniels, J , and Mackay, A L , *Nature*, **251**, 49–50 (1974).
5 May, R M., *Math. Biosci*, **12**, 59–79 (1971)
6 Somoraji, R L , and Goswami, D N , *Nature*, **236**, 466 (1972)
7 Saunders, P. T., and Bazin, M J , *J theor Biol* (in the press)

Plant growth substances and effects of photoperiod on flower bud development in *Phaseolus vulgaris*

THE flower buds of certain varieties of *Phaseolus vulgaris* absciss in British summers, a phenomenon which is induced when the plants are grown in day lengths longer than 13–14 h[1]. The number of buds which absciss increases as the day length is extended[1-3]. This post-initiation effect is truly photoperiodic, the photoperiodic stimulus being received in the leaves and the inhibitory effects expressed in the buds, the growth of which is inhibited before they finally drop[2-3]. Experiments in which a single trifoliate leaf was exposed to a photoperiod different from the rest of the plant provided indirect evidence that the effects of photoperiod on flower bud development were mediated through the production of substances in the leaves, with a predominance of inhibitor(s) being formed in long days and of promoter(s) in short days[3,4]. We describe here the determination in a single sensitive variety, classified as P47, of the chemical basis for

the inhibitory and promotory effects of long and short days respectively.

Exogenous application of either indolylacetic acid or gibberellic acid to the leaves or flower buds of plants kept in short days failed to induce the bud abscission experienced in long days[3,4]. In contrast, however, it has now been found[6] that application of synthetic abscisic acid (ABA) to the differentiating flower buds of plants in short days results in inhibition and finally abscission of many of the buds at a later stage of development. For example, when ABA was applied in a droplet of solution daily for 7 d (range 0 1–5 4 μg per application), the extent of flower bud abscission over the whole plant depended on the amount of ABA applied, the effect being most marked on those buds carried on the terminal inflorescence and at the uppermost node. Endogenous ABA extracted from plants grown in long days and re-applied to plants in short days evoked effects similar to those of the synthetic compound. On the other hand, exogenous application of the chemically related inhibitor, xanthoxin, did not induce flower bud abscission.

In view of the above results, indicating the possible participation of ABA in the regulation of flower bud development in this variety, numerous experiments were carried out during the summers of 1970–72 to study the effects of photoperiod on the endogenous concentrations of ABA ABA, both free and bound, was extracted[7] from different organs and the concentrations estimated, mostly by wheat coleoptile bioassay[8], with some confirmed by gas liquid chromatography[9]. The two methods gave results of the same order whether the tissues contained low or relatively high concentrations of ABA; for example, two different samples of leaves gave results by bioassay and gas liquid chromatography, respectively, of 33.5 and 35.4 μg per kg fresh tissue, and 225 and 208 μg per kg fresh tissue, these figures all being averages of several complete analyses of each extract.

Our experiments showed that the leaves and buds of plants grown in long days contained more ABA than those in short days (Table 1). The concentrations of ABA in the leaves, buds, petioles and stems of plants grown in an 18 h photoperiod (in which the buds were severely inhibited) were higher than those in the corresponding organs of plants grown in an 8 h photoperiod (in which bud development was normal). These differences in ABA concentrations were large in the youngest leaves but smaller in the mature leaves. The highest concentrations of ABA were found in the inhibited buds; these buds also contained considerable amounts of bound ABA.

Table 1   Concentration of ABA (μg per kg fresh tissue) in *Phaseolus vulgaris*, var. P47, grown in different photoperiods

Organ	Photoperiod 8 h	18 h
First trifoliate leaves	42	51
Second trifoliate leaves	21	37
Third trifoliate leaves	39	55
Fourth trifoliate leaves	30	91
Fifth trifoliate leaves (unfolding)	20	305
Combined petioles	11	36
Stems	18	29
Terminal flower buds	121 (9)*	729 (60)*

* Bound ABA in parentheses

Seeds were sown on May 5, 1972. In any 24 h periods all plants received 8 h natural daylight in a greenhouse, followed by either 0 or 10 h incandescent light (540 lx at pot level) in two photoperiod rooms, each maintained at 20 $\pm$ 1 °C. Plants were collected on June 7 when the final trifoliate leaf was unfolding and the flower buds on the plants in the 8 h photoperiod were just about to open. The individual organs from the plants were combined and extracted, and determinations were carried out by wheat coleoptile bioassay.
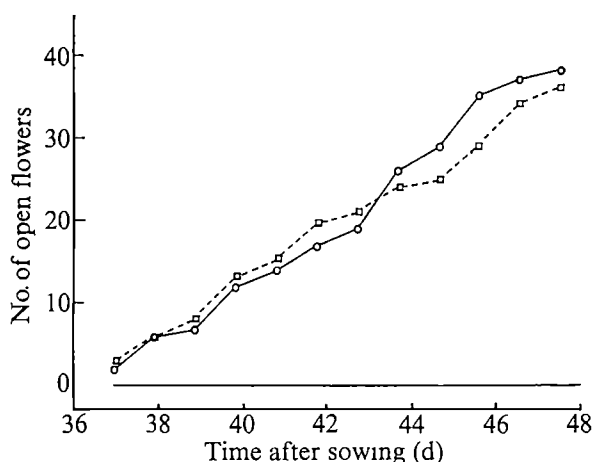
Fig. 1 Effect of cytokinins on the cumulative number of open flowers in long days. Plants were grown in a greenhouse for 8 h, extended to 16 h by incandescent light in a photoperiod room. From 15 d after sowing 10 μl solution containing 0.1 μg kinetin or 6-benzyl-aminopurine were added to the stem apex daily for 10 d. Data are totals of five plants. O, Kinetin; □, 6-benzyl-aminopurine. Control values remained at zero.

The above results, together with the effects of exogenously applied ABA, provide strong evidence that ABA plays an important role in mediating the effects of long days on flower bud development in variety P47. It would seem that under the influence of long photoperiods there is a greater production of ABA in the leaves and an increased accumulation of the substance in the buds, leading eventually to their inhibition and abscission. This was substantiated by a single experiment in which the abscissing buds were found to contain more free and bound ABA (331 and 70 μg per 100 g dry weight) than they did 6 d previously (105 and 0 μg per 100 g dry weight, respectively).

Evidence that promotory as well as inhibitory substances are involved in mediating the effects of photoperiod was obtained when cytokinins were applied to bean plants grown in long days[10]. Here it was found that kinetin or 6-benzyl-aminopurine stimulated the development of flower buds which would otherwise have dropped (Fig. 1) No study has yet been made on the endogenous concentrations of cytokinins in the bean variety P47; note, however, that the concentrations of cytokinins in both the leaves of *Begonia*[11] and the xylem sap of *Perilla*[12] were found to be higher in short days than in long days.

Previous work on the inhibitory and promotory effects of photoperiod on flowering in various species has concentrated on the process of floral initiation or more generally on flowering, and the identification of the plant growth substances involved has proved difficult[13]. The present study is therefore of particular significance in referring specifically to the effects of photoperiod on flower bud development after the initiation of the floral primordia, and provides evidence as to the identity of the inhibitory and promotory substances involved.

B. BENTLEY
CLARE B. MORGAN
D. G. MORGAN
F. A. SAAD

Department of Applied Biology,
University of Cambridge,
Cambridge, UK

1 Ojehomon, O. O., Rathjen, A. S., and Morgan, D. G., *J. agric. Sci.*, 71, 209–214 (1968).
2 Zehni, M. S., and Morgan, D. G., *Ann. Bot.* (in the press).
3 Zehni, M S , thesis, Univ. Cambridge (1969)
4 Zehni, M. S., Saad, F A , and Morgan, D G., *Nature*, 227, 628–629 (1970)
5 Ojehomon, O. O., Zehni, M. S , and Morgan, D G , *Ann. Bot.*, 37, 871–884 (1973)
6 Bentley, B., thesis, Univ. Cambridge (1974)
7 Milborrow, B. V , *J exp. Bot* , 21, 17–29 (1970).
8 Wright, S T. C., *Planta*, 86, 10–20 (1969).
9 Lenton, J. R., Perry, V M , and Saunders, P. F , *Planta*, 96, 271–280 (1971).
10 Saad, F A., thesis, Univ. Cambridge (1972).
11 Heide, O. M., and Skoog, F , *Physiol. Plant* , 20, 771–780 (1967)
12 Woolhouse, H. W , and Beever, J E , *Nature new Biol* , 246, 31–32 (1973)
13 Evans, L. T., *Induction of Flowering—Some Case Histories* (Macmillan, Melbourne 1969)

Suppression of apparent movement during binocular rivalry

STEREOSCOPIC depth perception can coexist with binocular rivalry of form and colour[1-3]. If a stereogram is viewed with a red filter in front of one eye and a green filter in front of the other eye (or with an oblique grating superimposed on one eye's picture and an orthogonal grating on the other picture), then stereopsis can still be obtained in spite of the two eyes' pictures being seen alternately rather than simultaneously. Although one image is 'suppressed' in consciousness, information about its position is preserved and used for stereopsis. This finding implies either that stereoscopic depth perception 'precedes' the site of binocular rivalry in the visual system or that conscious perception and stereopsis are independent visual functions mediated in 'parallel' neural channels. Here I consider the related question of whether an image that is suppressed during binocular rivalry (and is, therefore, not 'seen' by the observer) can contribute to the perception of apparent movement.

The display used in the experiment is illustrated in Fig. 1. Figure 1a and b were presented to the right and left eyes, respectively, in a three-channel tachistoscope driven by an electronic timer They subtended 9° and were of equal average luminance. Crossed polaroids were used to separate the two eyes' pictures The stimuli were high contrast horizontal (Fig 1b) and vertical (Fig 1a) gratings presented to one half-field alone in each eye. Figure 1a contained, in addition, a bright round yellow spot fixed to the centre of the grating. Figure 1c was displayed on the third channel either to the left eye alone (in one experimental condition) or to both eyes (in another condition), and contained only a yellow spot.

Four experienced observers were used in the experiment. They were instructed to press a button which illuminated Fig. 1a and b simultaneously These would stay on as long as the button was held pressed. When the button was released a and b would go off simultaneously and c would appear in the other half-field after a brief delay of 75 ms.

Initially b was excluded from the circuit and the observers familiarised themselves with apparent movement of the yellow spot between a and c by alternately pressing and releasing the button (the distance between these spots was about 3.5°). b was then included in the cycle. When the observers now pressed the button they could see rivalry between the striped half-fields in a and b. Sometimes periods of complete suppression would be observed and no yellow spot would be seen when the horizontal grating in the left eye b was suppressing the vertical grating in the right eye a. The observers were instructed to release the button during such periods of complete suppression of a and to observe whether the yellow spot in c seemed just to appear from nowhere or whether it seemed to move to its present location from its original (imaginary) location in a. They were encouraged to repeat the procedure as often as they liked before making a decision Specifically, they were asked to compare the effect of releasing the button during suppression of a, when the yellow spot was not visible, with the effect of releasing it during suppression of b.

All four observers reported that no apparent movement

Fig. 1 *a*, Right; *b*, left; *c*, left or both.
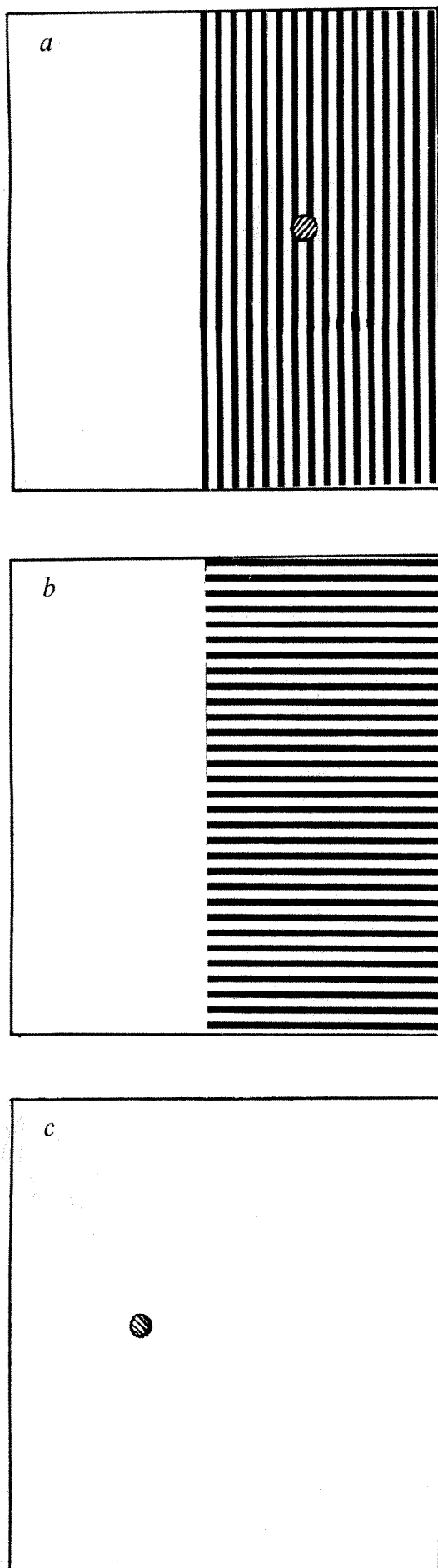
could be seen if the button was released when the yellow spot was being suppressed, suggesting that retinal rivalry precedes movement perception in the visual system. This was true irrespective of whether *c* was presented to the left eye alone or to both eyes. One of them, however, noted that if the button was released immediately following suppression then some vague impression of movement could, in fact, be obtained, presumably because the suppression itself took the place of switching off the light spot. If there was any delay between suppression and button release, then, like the other three observers, he saw no movement but only a single light spot *c* blinking on and off.

In this context it may be useful to think of apparent movement as an 'unconscious inference'[5-7] that a visible object has changed its location rather than as an expression of the time constants of hypothetical 'motion detectors'. The brain may judge it improbable that the disappearance of a stimulus should be followed so soon by the sudden and independent appearance of an identical stimulus nearby and may therefore decide that the original stimulus must in fact have moved to this new location. As conscious perception may be a necessary prerequisite for any cognitive inferential process of this nature it is perhaps not surprising that a suppressed and invisible image cannot contribute to apparent movement perception.

This view is not necessarily inconsistent with the ideas of Anstis[8], Julesz[9] and Braddick[10], as apparent movement is probably not a unitary process. The effects observed by these investigators using Julesz patterns may well be the result of activation of motion detectors at a relatively peripheral level in the nervous system. In fact Julesz[9] and Kolers[4] have argued before that motion perception may involve many different levels of processing.

The suppression of apparent movement during rivalry raises the related question of whether a single image can simultaneously contribute to stereopsis and apparent movement perception. To answer this question, the two half-images of a wheatstone stereogram were presented briefly to each eye alternately rather than simultaneously. (The interstimulus interval was 100 ms, and each stimulus lasted 100 ms.) If the disparity was large (1–2°) then the observers reported simultaneous stereo-depth and interocular horizontal apparent movement. This observation suggests that movement and depth perception are probably mediated in parallel neural channels with overlapping time constants.

I thank Dr O. Braddick and Alan Adlard for help and suggestions.

V. S. RAMACHANDRAN

Trinity College,
Cambridge, UK

1 Treisman, A., *Q. Jl exp. Psychol.*, **14**, 23 (1962).
2 Kaufman, L., *Am. J. Psychol.*, **77**, 193 (1964).
3 Ramachandran, V. S., Rao, V. M., and Vidyasagar, T. R., *Nature*, **242**, 412 (1973).
4 Kolers, P. A., *Vision Res.*, **12**, 1057 (1972).
5 Helmholtz, H. Von, *Treatise on Physiological Optics*, III (edit. by Southall, J. P. C. S.), (Dover, New York, 1962).
6 Ramachandran, V. S., Rao, V. M., and Vidyasagar, T. R., *Vision Res.*, **13**, 1399 (1973).
7 Sigman, E., and Rock, I., *Perception*, **3**, 9 (1974).
8 Anstis, S. M., *Vision Res.*, **10**, 1411 (1970).
9 Julesz, B., *Foundations of Cyclopean Perception* (University of Chicago Press, Chicago and London, 1971).
10 Braddick, O., *Vision Res.*, **14**, 519 (1974).

Immunobiology of membrane-bound collagen on mouse fibroblasts

THERE is ample evidence that collagen is produced by fibroblasts[1-5]. Following biosynthesis in ribosomes, collagen is thought to be transported to the periphery by microtubules[6] but very little is known about the biology of collagen in cell membranes. We report that collagen is membrane-bound, that it is patched and capped by anti-collagen sera, and that the
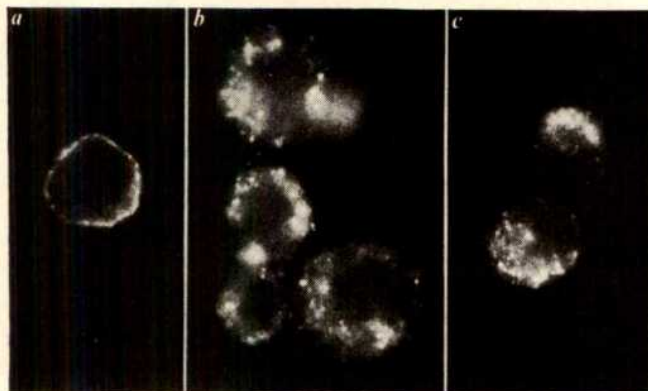
Fig. 1 Membrane immunofluorescence of mouse L-cell fibroblasts with rabbit anti-collagen serum. Reaction at 4° C (*a*), 22° C (*b*) and 37° C (*c*). Cells (1 × 10⁶) in 1 ml Eagle's MEM containing 3% FCS and HEPES buffer at specified temperatures were reacted with 30 μl of rabbit anti-rat skin collagen sera for 20 min, washed twice in medium, stained with fluorescein isothiocyanate (FITC) sheep anti-rabbit Ig (Wellcome, UK) for 20 min, washed three times in medium, suspended in 40% glycerol, and examined for membrane immunofluorescence in a Zeiss photomicroscope 2 equipped for epi-illumination[17]. Anti-collagen specificity was indicated for the antiserum according to the following major criteria: (1) by showing that the amino acid content of the antigen was consistent with collagen, and that the antigen contained no anti-rat or anti-mouse serum proteins using immunoelectrophoresis and Ouchterlony analysis; (2) by showing that the antiserum stained rat and mouse tissues in a collagen distribution using immunofluorescence according to Rothbard and Watson[16]; that the antiserum agglutinated collagen-coated sheep erythrocytes treated with tannic acid and that the staining of tissues and fibroblasts as well as the agglutination of red cells was removed by chromatography of the antiserum on rat collagen-coated beads of CnBr-activated sepharose or Degalan beads (Degussa, Frankfurt); (3) by showing that the antiserum did not precipitate rat or mouse serum proteins by immunoelectrophoresis or Ouchterlony analysis, and that it did not stain the cell membranes of mouse leukocytes or erythrocytes; (4) by showing that the antibody stained fibres in mouse tissues with collagen periodicity in the electron microscope using the antibody-peroxidase technique[25].

mobility of membrane-bound collagen is influenced by temperature and vinca alkaloids. The identification of membrane-bound collagen as a fibroblast marker would augment existing definitions of these cells, and would perhaps allow the application of certain immunological techniques to study collagen secretion by manipulating membrane-bound collagen with antibodies, lymphocytes, and so on.

Several proteins and receptors on fibroblasts have been studied immunologically. Histocompatibility antigens of mouse fibroblasts patch and cap with anti-H2 sera[7,8]; concanavalin A (con A) receptors on mouse 3T3 cells patch when treated with con A-ferritin complexes and anti-ferritin[9]; phytohaemagglutinin (PHA) receptors have been demonstrated on mouse L cells by agglutination and immunofluorescence techniques (A.T. and W.P.F., unpublished); and several types of mouse fibroblasts form spontaneous rosettes with erythrocytes[10,11]. The redistribution of these membrane structures are generally inhibited by low temperature or certain chemicals, usually vinca alkaloids. Although these reactions are specific, they are not unique to fibroblasts.

We used mouse (H2ᵏ) L-cell fibroblasts grown in Eagle's MEM supplemented with L-glutamine, 10% foetal calf serum (FCS) and 20 μg ml⁻¹ ascorbic acid. These cells were judged morphologically to be fibroblasts, and fixed cells were indirectly stained for collagen with anti-collagen serum[12]. The fibroblasts were grown to confluence in Falcon 3025 tissue culture flasks (75 cm²) and removed using either 0.2% trypsin for 10 min or 1% EDTA in phosphate buffered saline (PBS). After washing, the cells were suspended at a concentration of 1 × 10⁶ per ml in Eagle's MEM containing HEPES buffer and 3% FCS. Their

viability was greater than 99% as demonstrated by the exclusion of 0.1% trypan blue and by the intracellular hydrolysis of fluorescein diacetate[13]. These cells were then studied for membrane immunofluorescence using rabbit antisera to acid-soluble rat skin collagen. The antisera were prepared by first extracting an acid-soluble collagen antigen from rat skins with acetic acid according to the method of Bornstein and Piez[14]. The rat skin collagen, precipitated seven times, contained no rat serum proteins as demonstrated by immunoelectrophoresis against a potent, precipitating sheep antiserum to rat serum proteins, and the amino acid content of this antigen was consistent with collagen. Antisera were raised in rabbits immunised with 2 mg collagen in 1.5 ml Freund's adjuvant containing heat-killed *Mycobacterium tuberculosis*, followed 1 month later by 2 mg alum-precipitated[15] collagen intraperitoneally. A 1:100,000 dilution of the antisera agglutinated collagen-coated tanned sheep red blood cells, and this was inhibited by the addition of native rat or mouse skin collagens but not by native human skin collagen. The antisera did not precipitate rat or mouse serum proteins by immunoelectrophoresis. The sera produced a collagen pattern of staining[16] on cryostat sections of rat and mouse tissues but not on human tissues. The immunofluorescence in all of these experiments was carried out according to Faulk and Hijmans[17] (see Figs 1 and 2).

Viable L-cell fibroblasts produced a patchy, occasionally capped, pattern of membrane spots when stained at 22 °C with rabbit antisera to rat skin collagen (Fig. 1*b*). When this was performed at 4 °C the pattern of staining was homogeneous (Fig. 1*a*), and capped cells were seen when it was carried out at 37 °C (Fig. 1*c*). The transition from 4 °C to 37 °C (that is, transition of staining patterns from homogeneous to patch to cap) involved the coalescence of discrete membrane spots into a cap (Fig. 2). This transition did not occur in the presence of either colchicine or vinblastin, but was observed in the presence of cytochalasin B (Table 1). Cells treated at 37 °C with colchicine or vinblastin did not cap but had a characteristic pattern of peripheral staining. We were unable to observe a

Fig. 2 Coalescence of membrane-bound patches into a cap of collagen staining. L-cell fibroblasts treated with rabbit anti-collagen sera and FITC sheep anti-rabbit Ig. Control reagents for this experiment included (a) normal rabbit serum, normal human serum and normal sheep serum; (b) sheep anti-human normal serum and sheep anti-con A, and (c) FITC sheep anti-swine Ig and FITC rabbit anti-human Ig. All of these were negative. Arrows indicate the general direction of movement for patches of membrane-bound collagen to form a cap. We anticipate that, under suitable conditions, this is followed by secretion of collagen[11].
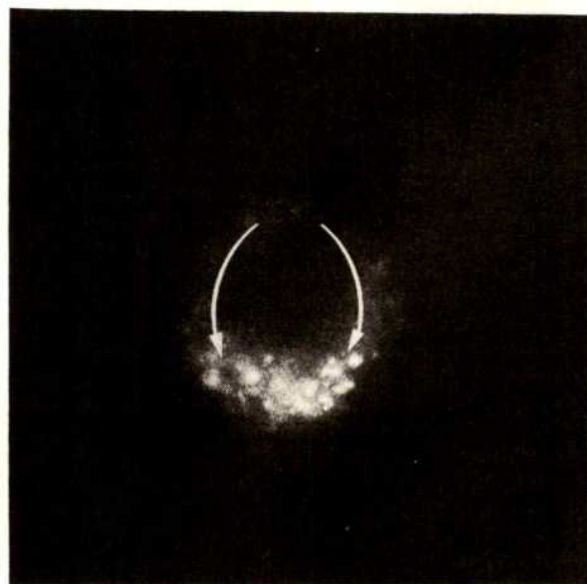
Table 1 Effect of temperature, cytochalasin B and vinca alkaloids on fibroblast patch and cap reactions with anti-collagen sera

Temperature	Reagent	Amount of reagent	Patch (%)	Cap (%)
4° C	PBS	—	5	none
37° C	PBS	—	>95	35
37° C	Cytochalasin	10 µg ml^{-1}	>95	37
37° C	Colchicine	10^{-5} M	>95	<5
37° C	Vinblastin	10^{-5} M	>95	<5

Cytochalasin B (Aldrich, UK), colchicine and vinblastin (Sigma, UK) were used. The cytochalasin B was dissolved in dimethyl sulphoxide at a concentration of 10 mg ml^{-1}. DMSO control had no observable effect on membrane immunofluorescence.

difference in collagen staining between cells collected using trypsin and EDTA.

Incubation of trypsin harvested cells for 1 h with 50 units of collagenase (BDH) under optimal conditions for the enzyme[1] still gave the usual staining pattern with anti-collagen sera. Subsequent incubation of collagenase-treated cells with 500 units of pronase (Koch-Light, *S. griseus*) for 1 h at 37 °C became negative for collegen staining. Pronase alone, however, was found to be responsible for this result. Pronase-treated cells giving no reaction for membrane-bound collagen become positive after 3 h incubation in complete medium containing ascorbic acid. The failure of collagenase to remove membrane-bound collagen is consistent with the observation that rabbit anti-collagen serum is primarily directed to terminal (telopeptide) antigenic sites, and not to helical or central sites[18].

Membrane-bound collagen may be unique to fibroblasts in a manner analogous to membrane-bound Ig being unique to B lymphocytes. This is not to imply that other cells cannot make collagen[19]. The membrane-bound collagen is mobile and temperature sensitive; its membrane mobility is partially inhibited by vinca alkaloids, suggesting a role for microtubules[20] in the mobility of membrane-bound collagen. The presence of membrane-bound collagen seems to be a useful criterion for defining fibroblasts and should be at least as accurate as currently existing morphological criteria. The membrane definition possibly has the additional advantage of defining subpopulations of fibroblasts in so far as collagens from different organs, such as skin or cartilage, are structurally different[21] and have different antigenicities[22]. These differences may be found in membrane-bound collagen, analogous to the phenomenon of allelic exclusion in B cells[23].

The presentation and mobility of collagen in the cell membranes of fibroblasts suggest several possible events in the biology of these cells. Capping of collagen may lead to the secretion of fibroblast cell product into the intracellular matrix. This theoretical consideration is strengthened by a recent observation that anti-fibroblast antibodies stimulate the secretion of trichloroacetic acid-precipitable cell product by fibroblasts[11]. Also, antibodies to chemically coupled substances on fibroblast cell membranes have been shown to cause a type of immunostimulation as measured by the incorporation of tritiated thymidine[24]. The presence of collagen in cell membranes could place fibroblasts at risk for immunologically mediated cytotoxic or suppressor reactions. We are studying these possibilities.

We thank Dr A. J. Bailey, ARC Meat Research Institute, Langford, Bristol for the collagen amino acid analysis.

W. PAGE FAULK
L. B. CONOCHIE
ANNE TEMPLE

MRC Rheumatism Unit,
Canadian Red Cross Memorial Hospital,
Taplow, Maidenhead, Berkshire, UK

M. PAPAMICHAIL

Department of Molecular Biology,
Hellenic National Research Institute,
Athens, Greece

1 Dehm, P., and Prockop, D. J., *Biochim. biophys. Acta*, 240, 358–369 (1971).
2 Layman, D. L., McGoodwin, E. B., and Martin, G. R., *Proc. natn. Acad. Sci. U.S.A.*, 68, 454–458 (1971).
3 Manner, G., *Expl Cell Res.*, 65, 49–60 (1971).
4 Levene, C. I., Bates, C. J., and Bailey, A. J., *Biochim. biophys. Acta*, 263, 574–584 (1972).
5 Steinberg, J., *J. Cell Sci.*, 12, 217–234 (1973).
6 Ehrlich, H. P., and Bornstein, P., *Nature new Biol.*, 238, 257–260 (1972).
7 Edidin, M., and Weiss, A., *Proc. natn. Acad. Sci. U.S.A.*, 69, 2456–2459 (1972).
8 Sundqvist, K. G., *Nature new Biol.*, 239, 147–149 (1972).
9 De Petris, S., Raff, M. C., and Mallucci, L., *Nature new Biol.*, 244, 275–278 (1973).
10 Elliott, E. V., Kerbel, R. S., and Phillips, B. J., *Nature*, 248, 514–515 (1974).
11 Faulk, W. Page, and Temple, A., *Regulation of Growth and Differentiated Function in Eukaryote Cells* (edit. by Talwar, G. P.), (Raven, New York, in the press).
12 Lustig, L., Costantini, H., and Mancini, R. E., *Proc. Soc. exp. Biol. Med.*, 130, 283–287 (1969).
13 Rotman, B., and Papermaster, B. W., *Proc. natn. Acad. Sci. U.S.A.*, 55, 134–141 (1966).
14 Bornstein, P., and Piez, K. A., *Biochemistry*, 5, 3460–3473 (1966).
15 Proom, H., *J. Path. Bact.*, 55, 419–426 (1943).
16 Rothbard, S., and Watson, R. F., *Lab. Invest.*, 27, 76–84 (1972).
17 Faulk, W. Page, and Hijmans, W., *Progr. Allergy*, 16, 9–39 (1972).
18 Timpl, R., Furthmayr, H., Hahn, E., Becker, U., and Stoltz, M., *Behring Inst. Res. Comm.*, 53, 66–79 (1973).
19 Perlman, M., Baum, J. M., and Kaye, G. I., *J. Cell Biol.*, 63, 306–311 (1974).
20 Allison, A. C., *Locomotion of Tissue Cells* (edit. by Porter, E. and Fitzsimons, D. W.), 109–148 (Elsevier, Amsterdam, 1973).
21 Gallop, P. M., Blumenfeld, D. D., and Seifter, S., *A. Rev. Biochem.*, 41, 617–672 (1972).
22 Hahn, E., Timpl, R., and Miller, E. J., *Immunology* 28, 561–568.
23 Froland, S. S., and Natvig, J. B., *J. exp. Med.*, 136, 409–414 (1972).
24 Shearer, W. T., Philpott, G. M., and Parker, C. W., *J. exp. Med.*, 139, 367–379 (1974).
25 Faulk, W. Page, Trencher, P., Darling, J., and Holborow, J., *Immunobiology of Trophoblast* (edit. by Edwards, R. G., Howe, C. W., and Johnson, H.), 113–130 (Cambridge University Press, Cambridge, 1975).

Endotoxin-sensitive membrane component of human platelets

GRAM-NEGATIVE bacteria produce endotoxin and cause intravascular coagulation, shock and ultimate death of an estimated 25% of patients with bloodstream infections[1,2]. The number of blood platelets in such patients decreases[3]. In animal experiments, endotoxin aggregates platelets, induces release of vasoactive amines and adenine nucleotides, and unmasks the phospholipid procoagulant (platelet factor 3, PF3), which accelerates blood clotting[4]. To determine the mechanism of endotoxin action in man we studied the endotoxin interaction with human platelets. Here we describe experiments which demonstrate that the endotoxin-sensitive component (receptor) is present on the membrane of human platelets. Two types of changes resulting from endotoxin interaction with this receptor have been noted: selective release of 5-hydroxytryptamine (5-HT) and unmasking of platelet phospholipid (PF3).

Human platelets rendered free of plasma proteins (see Table 1) were sensitive to endotoxin. Addition of endotoxin in the form of *Salmonella enteritidis* lipopolysaccharide B (Difco, Detroit, Michigan) at a concentration of 200 µg per ml human platelets caused release of ^{3}H-5-HT. In 21 separate experiments release of 5-HT ranged from 37% to 81% with a mean of 59±2.8%. This was significantly more ($P<0.01$) than release of 5-HT from human platelets incubated in the same conditions without endotoxin 21 ±0.8%). 5-HT release from the control platelets can be explained by the observation that newly absorbed 5-HT behaves usually as endogenous amine, although it has a tendency to be somewhat more preferentially released[7]. In the protein-free medium used here this phenomenon could be accentuated notwithstanding the fact that gel-filtered platelets seem to preserve their functional and ultrastructural integrity[8]. In these conditions the amount of 5-HT released by endotoxin consistently exceeded two or three times control values. Release of 5-HT was selective as the cytoplasmic marker enzyme, lactate dehydrogenase, was not liberated indicating that no significant lysis of human platelets occurred. The lysosomal marker enzyme, β glucuronidase was also not released. 5-HT release was, however, paralleled by release of adenine nucleotides, determined spectrophotometrically[9]. This suggests that platelet-dense granules containing 5-HT and adenine nucleotides were

Table 1 Effect of homogenate of human platelets or their membrane-rich preparation on endotoxin-induced ^{3}H-5-HT release from autologous platelets

Reaction mixture	% ^{3}H-5-HT release (mean ± s.e.m.)
Endotoxin + buffer + platelets	66.0±2.1
Endotoxin + homogenate* + platelets	46.6±2.8†
Endotoxin + MRP + platelets	45.7±0.7†
Buffer + homogenate + platelets	28.3±1.3
Buffer + platelets (control)	24.6±1.4

Human platelets, collected from fasting venous blood of healthy individuals who had not ingested aspirin or aspirin-like drugs during the preceding 8 d, were labelled with ^{3}H-5-HT added to platelet-rich plasma. Platelets were freed from plasma proteins and from unbound ^{3}H-5-HT by gel filtration according to the modified procedure of Tangen et al[5]. using a column containing Sepharose 2B. Platelets containing ^{3}H-5-HT were eluted in the void volume of the column and plasma proteins were distinctly separate from platelets in the elution profile. Separation of platelets from plasma proteins was further documented immunochemically using a panel of antisera to whole human serum, human gamma globulin, C3 component of complement and properdin factor B (C3 activator). Gel-filtered platelets gave negative results with these antisera in the rocket immunoelectrophoresis[6], whereas fractions containing plasma proteins were consistently positive.

*Homogenate was prepared by six cycles of freezing and thawing of gel-filtered human platelets not labelled with ^{3}H-5-HT.

MRP (membrane-rich pellet) was prepared from homogenate by centrifugation at 20,000g for 20 min and resuspending in 1 ml Tris-buffered saline pH 7.4. MRP was free of lysosomal β glucuronidase and lactate dehydrogenase, which were released to soluble fraction (supernatant).

Endotoxin (200 μg) was preincubated with 1 ml homogenate or MRP for 30 min at 37 °C and then gel-filtered autologous platelets labelled with ^{3}H-5-HT were added. After 30 min of incubation samples were centrifuged at 15,000g for 1 min at 0 °C and radioactivity in the supernatant was determined.

†Difference significant compared with reaction mixture containing endotoxin, buffer and platelets: $P < 0.01$.

selectively responding to endotoxin-induced membrane changes.

To establish that endotoxin effect on human platelets was specific, zymosan, another particulate material derived from yeast cell wall, and human erythrocyte stroma as a membrane particulate material were compared in concentrations similar to that of endotoxin. In contrast to endotoxin, neither zymosan nor red cell stroma caused a significant release of ^{3}H-5-HT from human platelets rendered free of plasma proteins. These data suggest that endotoxin has a specific affinity for the membrane of human platelets inducing a selective release reaction.

Experiments were carried out to determine whether the ability of human platelets to bind endotoxin plays a part in this interaction. Endotoxin was preincubated with a homogenate of unlabelled human platelets or with buffer. Then, fresh autologous human platelets labelled with ^{3}H-5-HT were added and ^{3}H-5-HT release was measured as an index of endotoxin activity. Human platelet homogenate significantly inhibited endotoxin interaction with intact platelets (Table 1) measured by release of ^{3}H-5-HT ($P<0.01$). When the membrane-rich fraction sedimented from human platelet homogenate was tested, a similar inhibition of endotoxin-induced release was observed. These experiments suggest that human platelets contain an endotoxin-binding component (receptor) associated with their membrane.

Interaction of endotoxin with human platelet receptor induced not only selective release of ^{3}H-5-HT but also unmasked platelet phospholipid (PF3), thus accelerating blood clotting. Evolution of PF3 activity in human platelets was measured by shortening of the Stypven time[10]. Human platelets were incubated for 30 min at 37 °C with endotoxin or with buffer and then the mixture was added to the autologous platelet-poor plasma in which Stypven time was measured. In three separate experiments endotoxin incubated with human platelets caused a significant ($P<0.01$)

shortening of the Stypven clotting time of human platelet-poor plasma (34.5±1.2 s) compared with plasma to which human platelets incubated without endotoxin were added (46.6±2.3 s).

As PF3 is associated with platelet membrane phospholipids[11] its evolution represents another example of platelet membrane-related biological activity of endotoxin.

Endotoxin seemed to interact directly with a sensitive component of human platelet membrane. Human platelets rendered free of immunochemically detectable plasma proteins, were treated with trypsin (Nutritional Biochemical, 2 μg ml^{-1}), known to destroy IgG and complement adsorbed to platelets[12,13]. This did not abolish the ability of human platelets to interact with endotoxin. Addition of heparin or hirudin as inhibitors of thrombin in concentration of 10 IU ml^{-1} did not interfere with endotoxin-induced release of ^{3}H-5-HT. This provided further evidence that adsorbed plasma proteins, if present, were not required in our experimental system. Second, experiments with neutralisation of the endotoxin effect by a membrane-rich preparation obtained from autologous human platelets suggest the existence of an endotoxin-binding component (receptor) on the human platelet membrane. When the receptor is triggered by endotoxin, release of vasoactive amine, 5-HT, and evolution of clot-promoting PF3 activity ensues. Thus, in addition to the well known indirect pathway of activation of human platelets by microbial antigens complexed with antibody and/or complement and interacting with platelets through receptors for IgG Fc fragment and complement[14,15], there is a direct pathway of human platelet activation as a result of the existence of a specific endotoxin-sensitive component (receptor) (Fig. 1).

Endotoxin-binding component (receptor) may be added to the list of previously reported receptors on the human platelet membrane. They include receptors for ADP (ref. 16), 5-HT (ref. 17), IgG fragment[9] and thrombin-sensitive protein[18]. The presence of an endotoxin-binding component on cells of human origin is not limited to the platelets. Endotoxin is known to interact directly, in the absence of serum, with polymorphonuclear leukocytes[19] and with red blood cells, to which it can be bound[20]. Endotoxin-sensitive component (receptor) on human platelets as demonstrated in our study is important as platelets are intimately involved in the activation of blood coagulation, and their interaction with endotoxin may help us to understand better endotoxin-induced intravascular coagulation and shock in man.

Fig. 1 Pathways of human platelet activation by endotoxin from Gram-negative bacteria. *a*, Direct pathway; *b*, indirect pathway.
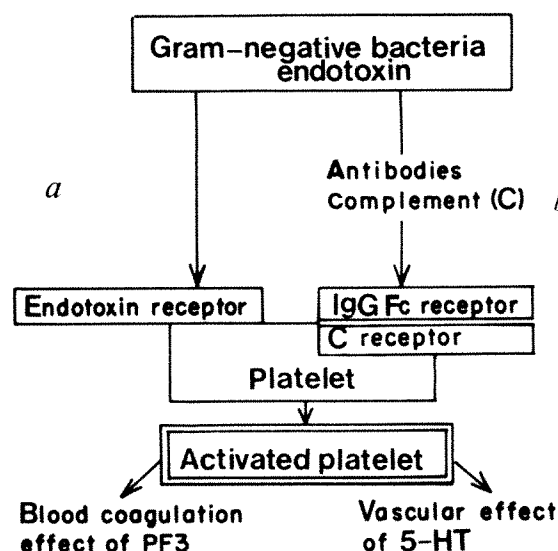
J. HAWIGER
A. HAWIGER
S. TIMMONS

Department of Medicine,
Vanderbilt University School of Medicine
and, Veterans Administration Hospital,
Nashville, Tennessee 37232

1 Minna, J. D., Robboy, S. J., and Colman, R. W., *Disseminated Intravascular Coagulation in Man*, 3–18 (Charles C. Thomas, Springfield, Illinois, 1974).
2 Wolff, S. M., and Bennet, J. V., *New Engl. J. Med.*, 291, 733–734 (1974).
3 Cohen, P., and Gardner, F. H., *Archs. intern. Med.*, 117, 113–124 (1966).
4 Des Prez, R. M., Horowitz, H. I., and Hook, E. W., *J. exp. Med.*, 114, 857–873 (1961).
5 Tangen, O., Berman, H. J., and Marfey, P., *Thromb. Diath. Haemorrh.*, 25, 268–278 (1971).
6 Laurell, C. B., *Analyt. Biochem.*, 15, 45–52 (1966).
7 Holmsen, H., Ostvold, A. C., and Day, H. J., *Biochem. Pharmac.*, 22, 2599–2608 (1973).
8 Hutton, R. A., Howard, M. A., Deykin, D., and Hardisty, R. M., *Thromb. Diath. Haemorrh.*, 31, 119–132 (1974).
9 Henson, P. M., and Spiegelberg, H, L., *J. clin. Invest.*, 52, 1282–1288 (1973).
10 O'Brien, J. R., *Nature*, 181, 420 (1958).
11 Marcus, A. J., Bradlow, B. A., Safier, L. B., and Ullman, H. L., *Thromb. Diath. Haemorrh. suppl.*, 26, 43–52 (1967).
12 Nachman, R. L., *Semin. Hematol.*, 5, 18–31 (1968).
13 Henson, P. M., *Immunology*, 16, 107–121 (1969).
14 Pfueller, S. L., and Luscher, E. F., *J. Immun.*, 112, 1201–1210 (1974).
15 Zucker, M. B., and Grant, R. A., *J. Immun.*, 112, 1219–1230 (1974).
16 Born, G. V. R., *Ann. R. Coll. Surg.*, 36, 200–205 (1965).
17 Michal, F., *Nature*, 221, 1253–1254 (1969).
18 Baenzinger, N. L., Brodie, G. N., and Majerus, P. W., *Proc. natn. Acad. Sci. U.S.A.*, 68, 240–243 (1971).
19 Cohn, Z. A., and Morse, S. I., *J. exp. Med.*, 111, 689–704 (1960).
20 Young, V. M., Gillem, C. H., and Ackeroyd, J. V., *J. Pediat.*, 60, 172–176 (1962).

Characterisation of the bacteriophage T4 receptor site

THE fundamental importance of specific cellular interaction is indicated by its ubiquity in biological systems. Such specificity must be mediated by macromolecular recognition of intrinsically the same nature as enzyme–substrate binding and antigen–antibody interactions, but few systems involving recognition between two cells or two organisms are readily amenable to investigation at the molecular level. The attachment of bacteriophage to its host is an exception, and the chemical constitution of the specific receptor site involved in the initial binding of phage T4 to *Escherichia coli* B has therefore been examined.

The T-even bacteriophages adsorb to their host bacteria initially by the tail fibres[1], and there are strong indications that this interaction determines the specificity of adsorption and thus delineates the possible host range of the phage[2-4]. The initial receptor site for phage T4 is situated in the host lipopolysaccharide[5-7], but apart from the demonstration that T4-resistant *E. coli* mutants lack UDPG pyrophosphorylase[8,9] the receptor has not been characterised further. The *E. coli* B lipopolysaccharide consists only of the core region, and a recent analysis[10] indicates that in this strain the major carbohydrate components of the core lipopolysaccharide are heptose, glucose and glucosamine; some pentose was also detected. An assay was devised to determine which of these components acts as receptor site for phage T4, and its linkage to the rest of the lipopolysaccharide.

The interaction of T4 with washed *E. coli* B cells was determined in the simplest medium which permits adsorption, in the presence of a variety of mono-, di- and tri-saccharides. Any sugar mimicking the whole or part of the phage receptor site should inhibit adsorption of the phage to the cell wall. Sugars unrelated to the receptor would have little effect. A similar approach has been used in studying the antigenic determinants of complex polysaccharides, but it has not hitherto been applied to analysis of specific interactions between organisms.
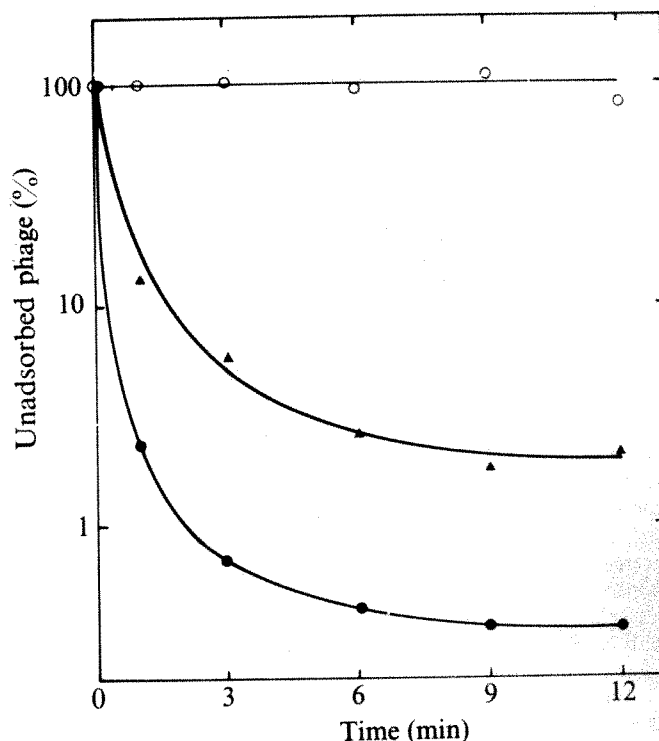
Fig. 1 Effects of D-glucose and D-fructose on bacteriophage T4 adsorption to *E. coli* B. Washed bacteria (5×10^8 cells ml^{-1}) and phage T4 (10^7 phage ml^{-1}) were mixed in 0.01 M Tris buffer, pH 7.8, containing 0.1 M NaCl at 30 °C. At intervals samples were centrifuged to remove bacteria and unadsorbed phage titred. ●, No sugar added; ○, 0.6 M D-glucose added; ▲, 0.6 M D-fructose added.

The effects of fructose, which is not a component of the *E. coli* cell wall, and glucose on adsorption of T4 are illustrated in Fig. 1. Glucose completely inhibits the adsorption, while the effect of fructose is much less dramatic and apparently nonspecific. Glucose or a related molecule therefore constitutes an essential part of the phage receptor site.

In order to determine the exact structural specifications at this site, the effects on phage adsorption of some of the epimers and derivatives of glucose were examined (Table 1). The ineffectiveness of mannose as an inhibitor indicates that the configuration at C-2 must be specifically that of glucose. Similarly the inability of galactose to inhibit binding demonstrates the importance of the configuration at C-4. Xylose had no effect on adsorption and although glucuronic acid did inhibit binding it was less effective than glucose, suggesting an absolute requirement for the C-6 group and a preference for —CH$_2$OH rather than —COOH at this position. Substitution or replacement of the glucose hydroxyl groups, unlike configurational change, frequently gave molecules which were effective antagonists of phage binding. This is true of the C-1, C-2 and C-3 positions, for glucosamine, N-acetylglucosamine, 2-deoxyglucose, 3-O-methylglucose and 1 : 5-gluconolactone all completely inhibited adsorption. Thus an aldohexose with configurations at C-2 and C-4 which are specifically those of glucose is the essential unit constituting the phage T4 receptor site. Glucose is the only naturally occurring sugar in this configuration, and it may be present as the hexose, glucosamine or N-acetylglucosamine, or as some more unusual derivative substituted at C-1, C-2 or C-3.

Although glucose is probably a component of all lipopolysaccharide molecules[11], bacteriophage T4 has an extremely narrow host range and does not adsorb to many Gram-negative organisms otherwise capable of supporting its growth[12]. On the basis that this specificity probably arises from the presence of a particular linkage in the lipopolysaccharide, the effects of a variety of di- and tri-saccharides

Table 1 Effects of mono-, di- and trisaccharides on adsorption of phage T4 to *E. coli* B

Monosaccharide	Inhibition of adsorption (%)	Di- or trisaccharide	Inhibition of adsorption (%)
D-Glucose	100	Sucrose	100
D-Galactose	< 1	Trehalose	100
D-Mannose	< 1	Maltose	100
D-Xylose	< 1	Turanose	100
D-Glucosamine	100	Kojibiose	100
N-Acetyl-D-glucosamine	100	Cellobiose	< 1
2-Deoxy-D-glucose	100	Lactose	< 1
3-O-Methyl-D-glucose	100	Melezitose	100
1 : 5-D-Gluconolactone	100	Raffinose	< 1
D-Glucuronic acid	10		

Adsorption curves (Fig. 1) were determined in the presence of 0.6 M sugar, after readjustment of pH to 7.8 when necessary. In all cases a plateau was reached between 6 and 12 min, and inhibition of adsorption was calculated from the number of unadsorbed phage at 12 min.

containing glucose were investigated (Table 1). Only those sugars in which glucose is α-linked to another residue inhibited adsorption; a β-linkage at C-1, as in cellobiose, rendered the molecule inactive. Moreover, linkage of a sugar residue to the C-4 or C-6 position of the glucose component in lactose and raffinose also destroyed activity. It appears that the glucose or glucose derivative acting as receptor for T4 must be α-linked to the rest of the lipopolysaccharide and cannot be bonded to another sugar residue through either the C-4 or C-6 hydroxyl groups.

Inhibition of binding should be more effective the closer the structure of the sugar inhibitor is to that of the receptor. Sugars which inhibit binding fell into three classes in terms of the dependence of degree of inhibition on sugar concentration (Fig. 2). All monosaccharides tested—glucosamine, N-acetylglucosamine, 2-deoxyglucose, 3-O-methylglucose and 1 : 5-gluconolactone—displayed the same concentration effect as glucose; the dependence of inhibition on sugar concentration did not therefore indicate whether glucose or one of its derivatives was the basic component of the T4 receptor site. Maltose was slightly more effective, and sucrose, turanose, kojibiose, trehalose and melezitose all inhibited binding at significantly lower concentrations than glucose. It seems that the nature and number of the sugars to which glucose is linked do not affect its activity as an inhibitor, but that the α1→4 linkage of glucose to glucose in maltose results in a less favourable configuration of the

inhibitor than the α1→1 or α1→2 linkage of glucose to glucose and the α1→2 or α1→3 linkage of glucose to fructose found in more effective inhibitors. Apparently, therefore, the glucose, glucosamine or closely related residue which acts as the T4 receptor may be in a terminal position α-linked to the 2- or 3-position of the adjoining residue, or it may be internally situated in the carbohydrate chain through linkages to its own C-2 or C-3 position. Determination of the exact structure of the receptor in *E. coli* B awaits sequencing of its lipopolysaccharide, but the value of this type of technique for partial determination and comparison of both phage receptors and bacterial surface structures is evident, as is its possible application to a wide variety of problems of intercellular recognition.

JOAN DAWES

Department of Microbiology,
University of Edinburgh,
West Mains Road,
Edinburgh EH9 3JG, UK

1 Simon, L. D., and Anderson, T. F., *Virology*, 32, 279–297 (1967).
2 Williams, R. C., and Fraser, D., *Virology*, 2, 289–307 (1956).
3 Kellenberger, E., and Séchaud, J., *Virology*, 3, 256–274 (1957).
4 Wildy, P., and Anderson, T. F., *J. gen. Microbiol.*, 34, 273–283 (1964).
5 Weidel, W., Koch, G., and Lohss, F., *Z. Naturforsch.*, 9b, 398–406 (1954).
6 Goebel, W. F., and Jesaitis, M. A., *J. exp. Med.*, 96, 425–438 (1952).
7 Wilson, J. H., Luftig, R. B., and Wood, W. B., *J. molec. Biol.*, 51, 423–434 (1970).
8 Symonds, N., Stacey, K. A., Glover, S. W., Schell, J., and Silver, S., *Biochem. biophys. Res. Commun.*, 12, 220–222 (1963).
9 Hattman, S., and Fukasawa, T., *Proc. natn. Acad. Sci. U.S.A.*, 50, 297–300 (1963).
10 Boman, H. G., and Monner, D. A., *J. Bact.*, 121, 455–464 (1975).
11 Rapin, A. M. C., and Kalckar, H. M., *Microbial Toxins IV* (edit. by Weinbaum, G., Kadis, S., and Ajl, S. J.), 267–307 (Academic Press, New York, 1971).
12 Wais, A. C., and Goldberg, E. B., *Virology*, 39, 153–161 (1969).

Fig. 2 Dependence of adsorption inhibition on sugar concentration. Adsorption curves (Fig. 1) were determined at different sugar concentrations, and inhibition of adsorption calculated from the number of unadsorbed phage at 12 min (Table 1). ○, D-glucose; D-glucosamine, N-acetyl-D-glucosamine, 2-deoxy-D-glucose, 3-O-methyl-D-glucose and 1 : 5-D-gluconolactone also gave this concentration curve. ●, Maltose; ▲, sucrose; the same curve was obtained with turanose, kojibiose, trehalose and melezitose.
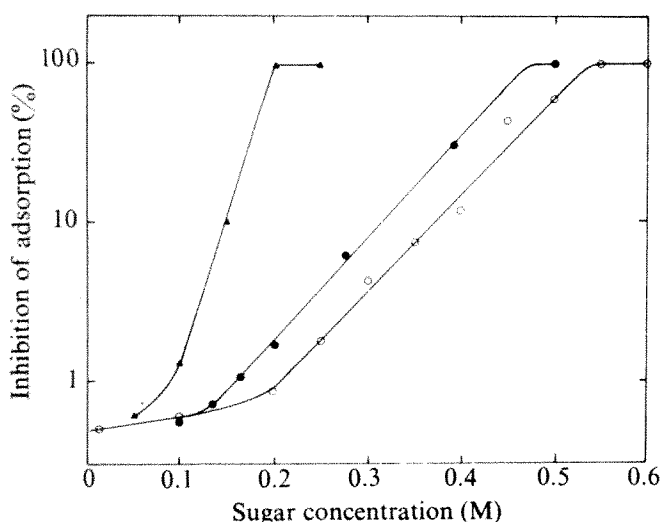
Experimental autoimmune myasthenia induced in monkeys by purified acetylcholine receptor

MYASTHENIA GRAVIS (MG) is a neuromuscular junction disease of man characterised by muscular weakness and fatigability.

Evidence that an autoimmune response to the postsynaptic nicotinic acetylcholine receptor (AChR) is important in the pathogenesis of MG has recently been presented. We have demonstrated a specific sensitisation of lymphocytes from MG patients when cultured *in vitro* with an AChR fraction extracted from the electric organ of the electric eel[1]; we have further shown reduction of such sensitisation in lymphocytes from patients under steroid treatment displaying clinical improvement[2]. Fambrough *et al.*[3] have shown that there is a reduction in the number of junctional acetylcholine receptors in myasthenic muscles. Patrick and Lindstrom[4], Heilbronn *et al.*[5] and Aharonov *et al.*[6] induced experimental autoimmune myasthenia in rabbits by injection of AChR isolated from electric organ
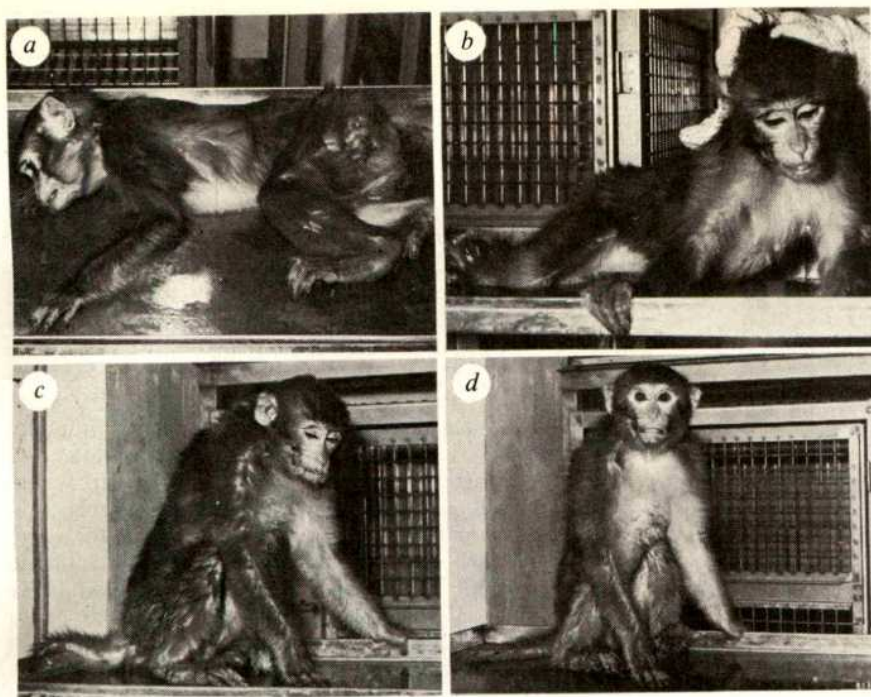
Fig. 1 Myasthenic monkey one week after fourth injection of purified acetylcholine receptor. *a, b*, Before injection of Prostigmine; *c*, 5 min after injection of Prostigmine (0.2 mg); *d*, 10 min after injection of Prostigmine.

tissue of either the electric eel, *Electrophorus electricus* or the electric ray, *Torpedo*.

Here we describe the induction of experimental auto-immune myasthenia gravis in Rhesus monkeys by injection of AChR purified from the electrogenic tissue of *T. californica*. The clinical, pharmacological electrophysiological, immunological and histological findings in monkeys suffering from experimental autoimmune myasthenia closely parallel those in patients suffering from MG.

Purified AChR from electrogenic tissue of *T. californica* was obtained by solubilisation of membrane fragments with 1% Triton X-100, followed by affinity chromatography on a *Naja naja siamensis* neurotoxin–Sepharose resin, as described by Aharonov *et al.*[6], with slight modifications, for isolation of AChR from the electric eel. The specific activity of the purified AChR was 8,000–10,000 pmol toxin bound per mg protein.

Two female Rhesus monkeys, *Macaca mulatta* (3 kg), were injected in both foot pads and intradermally in 3–4 spots with 100–200 µg AChR in 0.5 ml saline mixed with 0.7 ml Freund's complete adjuvant (FCA). At three-week intervals, both animals were subsequently challenged three times with AChR administered in the same way. Control monkeys received FCA alone. Within two weeks of the third injection of AChR, the two monkeys showed signs of fatigue, hypoactivity, anorexia and weight loss. One week after the fourth injection the monkeys' condition was acute, with extreme flaccid paralysis of the limbs and trunk, sinking of the head and severe difficulties in breathing (Fig. 1*a* and *b*). Bulbar signs included ptosis, external ophthalmoplegia, facial diplegia and paralysis of the jaw muscles, with uncontrolled salivation and severe dysphagia. Shortly after intramuscular injection of 0.2 mg of the anticholinesterase Prostigmine (neostigmine bromide) the sick monkeys began to display signs of recovery which was complete within ten minutes: they became erect, raised their heads, placed their legs under their bodies, were able to move and essentially to breathe normally; at the same time, all bulbar signs disappeared (Fig. 1*c* and *d*). This improved condition was maintained for about 4 h. No clinical changes were observed in monkeys injected with FCA alone.

Neurophysiological studies were made on the animals after 6 weeks, before the fourth injection, using a Grass electromyograph with stimulator unit and differential amplifier. A coaxial recording electrode was introduced into the calf muscle and a bipolar stimulation electrode was placed on the buttock over the sciatic nerve. The electromyographic examination revealed a decrease in amplitude during repetitive nerve stimulation in AChR-injected monkeys (Fig. 2*a*) and a normal response in control animals (Fig. 2*b*).

After the pharmacological investigations and before the animals were killed, blood was collected by heart puncture and humoral antibodies against purified AChR were detected by the agar gel immunodiffusion assay in the sera of the animals (see Fig. 3). Cellular sensitivity towards

Fig. 2 Electromyographs from myasthenic and normal monkeys. Tetanic stimulation was carried out at a frequency of 40 s^{-1}, with pulses of 0.1 ms duration and 150 V amplitude. Horizontal bar, 2 s; vertical bar, 5 mV. *a*, Myasthenic monkey; *b*, Control monkey.
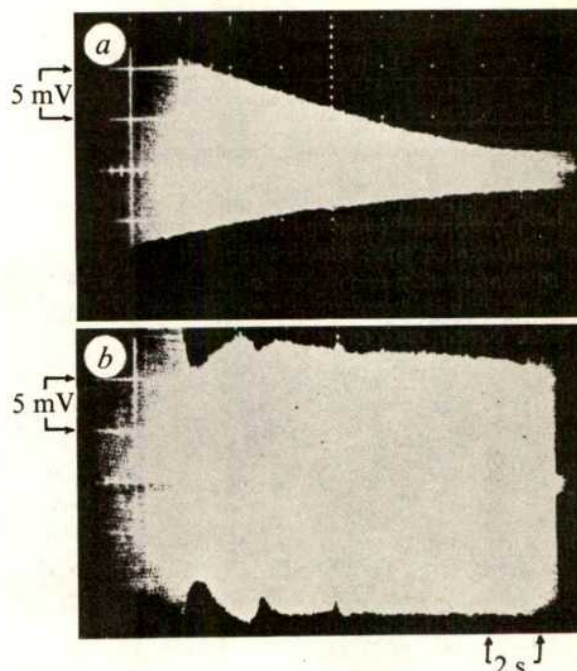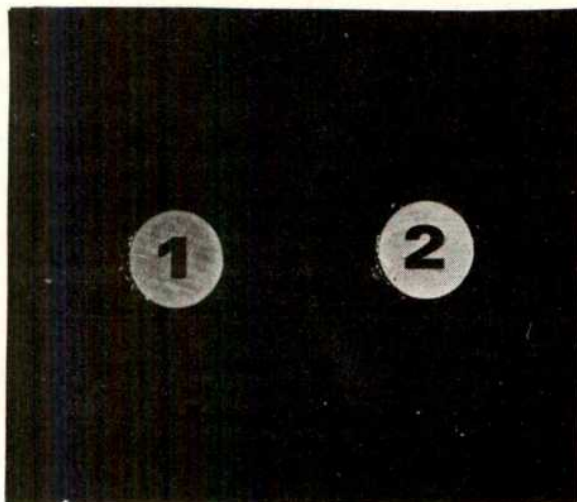
Fig. 3 Agar gel immunodiffusion of serum from a sick monkey. Well 1, monkey serum; well 2, purified acetylcholine receptor (300 μg ml^{-1}).

AChR was demonstrated in lymph node cells using the lymphocyte transformation technique (2-^{14}C-thymidine uptake) according to the method described by Tarrab *et al.*[7]. The results are expressed as stimulation indices (SI) (the ratio of radioactivity in tubes containing antigen to the radioactivity in control antigen-free tubes), and they represented an average of values obtained in duplicate cultures. Lymphocytes of myasthenic monkeys showed SI of 3.0, 12.5 and 12.8 when incubated in the presence of 0.5 μg, 1 μg and 2 μg AChR, respectively, and SI of 0.8–1.4 when incubated with other antigens such as rabbit actin, rat collagen and lysozyme. No stimulation was obtained in the control animals with either AChR (SI 0.9–1.3) or the other antigens (SI 0.8–1.4).

Samples of brain, thymus and muscles were taken for pathological examination. The formalin-fixed paraffin-embedded sections were stained with haematoxylin–eosin and light green. The histopathology revealed a few lymphocytes between muscle cells in the leg muscle and mild lymphorrhage foci in a buccal muscle (Fig. 4).

The clinical picture and the histopathological findings, as well as the characteristic clinical improvement after anti-cholinesterase administration and the pathognomonic decremental response to repetitive nerve stimulation, indicate that the monkeys suffered from myasthenia. The criteria for diagnosis of MG in patients suffering from criteria applied are closely analogous to the principal muscular weakness.

Fifteen years ago it was already suggested that MG

Fig. 4 Lymphorrhage focus in a buccal muscle section from a myasthenic monkey. Haematoxylin–eosin and light green. ×280.

might be an autoimmune disease of the neuromuscular junction[8,9]. Goldstein and coworkers[10], Kalden *et al.*[11] and Kawanami and Mori[12] attempted to provide an experimental model for the disease in experimental animals injected with either muscle or thymic extracts. Although electrophysiological evidence for a partial neuromuscular block was demonstrated, the animals did not show any clinical signs of muscle paralysis, and others were unable to confirm these findings[13–15]. Clinical as well as electrophysiological and pharmacological signs of myasthenia were recently induced by Patrick and Lindstrom[4] and subsequently by Heilbronn *et al.*[5] and ourselves[6] in rabbits immunised with AChR purified from electric fish. The nicotinic AChR of the electrogenic organ tissue of the electric fish is pharmacologically similar to the same receptor in skeletal muscle[16], and cross reacts immunologically with AChR from other species[4,17] and, as the present study suggests, most probably with monkeys as well. These findings thus provide supporting evidence that MG is an autoimmune disease and that AChR is the autoantigen. Our earlier observations[1,2] on a cellular immune response to AChR of lymphocytes from patients with MG provided direct evidence that breakdown of tolerance to self-AChR is involved in the neuromuscular block. The AChR-induced myasthenia in monkeys, so similar to the human disease, provides a valuable experimental model for studying both the mechanism and therapy of myasthenia gravis.

REBECA TARRAB-HAZDAI
AHARON AHARONOV
ISRAEL SILMAN
SARA FUCHS

Departments of Chemical Immunology and Biophysics, The Weizmann Institute of Science, Rehovot, Israel

ODED ABRAMSKY

Department of Neurology, Hadassah University Hospital, Jerusalem, Israel

1 Abramsky, O., Aharonov, A., Webb, C., and Fuchs, S., *Clin. exp. Immun.*, **19**, 11–16 (1975).
2 Abramsky, O., Aharonov, A., Teitelbaum, D., and Fuchs, S., *Archs Neurol.* (in the press).
3 Fambrough, D. M., Drachman, D. B., and Satyamurti, S., *Science*, **182**, 293–295 (1973).
4 Patrick, J., and Lindstrom, J., *Science*, **180**, 871–872 (1973).
5 Heilbronn, E., Mattson, C., Stalberg, E., and Hilton-Brown, P., *J. neurol. Sci.*, **24**, 59–64 (1975).
6 Aharonov, A., Kalderon, N., Silman, I., and Fuchs, S., *Immunochemistry* (in the press).
7 Tarrab, R., Sulica, A., Haimovich, J., and Sela, M., *Eur. J. Immun.*, **1**, 231–236 (1971).
8 Simpson, J. A., *Scot. Med. J.*, **5**, 419–436 (1960).
9 Nastuk, W. L., Plescia, O. J., and Osserman, K. E., *Proc. Soc. exp. Biol. Med.*, **105**, 177–184 (1960).
10 Goldstein, G., Strauss, A. J. L., and Pickeral, S., *Clin. exp. Immun.*, **4**, 3–16 (1969).
11 Kalden, J. R., Williamson, W. G., Johnston, R. J., and Irvine, W. J., *Clin. exp. Immun.*, **5**, 319–340 (1969).
12 Kawanami, S., and Mori, R., *Clin. exp. Immun.*, **12**, 447–454 (1972).
13 Vetters, J. M., Simpson, J. A., and Folkarde, A., *Lancet*, i, 28–31 (1969).
14 Kaufman, B. M., Rushworth, G., and Wright, R., *J. neurol. neurosurg. Psychiat.*, **32**, 281–289 (1969).
15 Jones, S. F., Brennan, J. L., and McLeod, J. G., *J. neurol. neurosurg. Psychiat.*, **34**, 399–403 (1971).
16 Hall, Z. W., *A. Rev. Biochem.*, **41**, 925–952 (1972).
17 Sugiyama, H., Benda, P., Meunier, J. C., and Changeux, J. P., *FEBS Lett.*, **35**, 124–128 (1973).

Anti-fertility drugs: novel non-hormonal compounds that inhibit prostaglandin metabolism

PREGNANCY has been terminated in animals and/or humans by oestrogens[1–3], androgens[4,5], anti-oestrogens[6,7] prostaglandins[8–10], and various non-steroidal non-hormonal agents[6–11].

Table 1 Subcutaneous doses of L10492 and L10503 for each of 5 consecutive days required for termination of pregnancy in 100% rats and hamsters

Animal	Treatment on gestation days	L10492	L10503
		(mg kg^{-1} d^{-1})	
Rat	1–5	25.0	100.0
	4–8	10.0	30.0
	6–10	2.5	20.0
	8–12	10.0	30.0
Hamster	1–5	2.0	0.5
	4–8	0.5	0.25
	6–10	3.0	1.5
	8–12	>10.0	>5.0

The mechanism of action of these agents has not been defined and effectiveness varies with the species. Elevated levels of endogenous prostaglandins (PGs) may be associated with toxaemia of pregnancy and habitual abortion[12]. PG may also alter foetal and placental blood flow[13,14]. A compound that inhibits PG metabolism—thereby elevating PG levels—could possibly interrupt pregnancy. This report describes a new class of non-hormonal compounds, which are 100% effective as anti-fertility agents in several species of animals, and also outlines studies on the effect of these compounds on PG synthesis and metabolism in rat placenta.

Compounds studied were 2-(3-methoxyphenyl)-5-H-s-triazolo [5,1-α] isoindole (L10492) and 2-(3-methoxyphenyl)-5,6-di-hydro-s-triazolo [5,1-α] isoquinoline (L10503). These compounds in sesame oil were administered subcutaneously to female Sprague–Dawley rats and Golden Syrian hamsters on various days after mating, the rats and hamsters were killed on days 16 and 14 of pregnancy, respectively, and the uteri examined for live or dead foetuses, implantation sites or resorpting foetuses.

Dose-related anti-fertility effects were obtained with both compounds when administered in various 5 d segments during the first half of gestation in both species (Table 1). The most effective time for treatment was during gestation days 6–10 in rats and days 4–8 in hamsters. Although 100% pregnancy termination was obtained in each treatment period, the most effective time for treatment was soon after implantation of the fertilised ova. Note that L10492 was more potent in rats and that L10503 was more potent in hamsters. The latter, however, was more sensitive to treatment with either compound. Single injections of these compounds also produced dose and time of pregnancy-related abortifative activity in both species (Fig. 1). Dose required for 100% effectiveness, however, was higher than that needed with multiple injections. The optimum time for drug administration was during days 4 or 5 in the hamster and days 7 or 8 in the rat, corresponding to the time immediately following implantation of the blastocyst in the uterus. As in the case of the multiple injection studies, the hamster was the more sensitive species and L10492 was more potent in the rat than L10503; the latter was, however, more potent in the hamster.

Studies on the mechanism of action indicated that the primary site for drug action is at the utero–placental complex. Higher brain centres, hypothalamus, pituitary and ovaries are not required for the anti-fertility activity as rats ovariectomised on day 8 of pregnancy and maintained in the gravid state by daily subcutaneous injections of 10 mg progesterone plus 1 μg oestradiol benzoate, resorbed all their foetuses after a single 50 mg kg^{-1} injection of L10492. Similar results were obtained in both animal species with both compounds in several different treatment regimens. In addition pregnant rats administered single (days 7 or 8 of pregnancy) or multiple (days 6–10 of pregnancy) doses of L10492 (50 mg kg^{-1} single or 2.5 mg kg^{-1} d^{-1}) or L10503 (100 mg kg^{-1} single or 25 mg kg^{-1} d^{-1}) and progesterone at daily doses of 4 mg per rat on days 6–15 of gestation, resorbed the products of pregnancy. These treatments did not affect the corpora lutea which were well developed

and apparently functional during foetal resorption. Mechanism of action does not involve ovulation inhibition as treatment was initiated after ovulation and conception (Table 1 and Fig. 1). Also rats and hamsters treated with abortifative doses of each compound in various phases of their oestrous cycles ovulated normally. These compounds did not inhibit implantation of the fertilised ova as administration of L10492 or L10503 after ovulation and mating but before implantation allowed the ova to implant but subsequently the implants were resorbed (Table 1 and Fig. 1). Hormonal or anti-hormonal activity is not involved in the anti-fertility action of these agents as they are devoid of oestrogenic, anti-oestrogenic, androgenic, anti-androgenic, progestational or anti-progesterone activities.

The possibility that placental PG synthesis and metabolism was altered by drug treatment was investigated. Metabolism was studied as described previously[15]. Synthesis was studied using the method of Flower et al.[16] and modified as described in Table 2.

Placental PG metabolism varies with the day of pregnancy and is lowest on day 8, is sharply increased on day 11, returns to a low level on day 14 and then becomes maximal on days 16 to parturition. As the optimum time for anti-fertility activity of L10503 (100 mg kg^{-1}, subcutaneously) was found to be on days 7, 8 or 9 of gestation[17], we studied the effect on placental PG metabolism on days 8, 9 and 10 and on PG synthesis on day 9. L10503 inhibited PG metabolism on a per mg protein basis, the degree of inhibition being dependent on the day of drug treatment. In the case of PGE_1 there was no alteration in metabolism when the compound was administered on days 8, 9 or 10 of gestation but when treatment was on both days 9 and 10 there was a 48% inhibition of metabolism. $PGF_{2\alpha}$ metabolism, however, markedly decreased when L10503 was injected on days 8, 9 or 9 and 10 but a single injection on day 10 produced no significant change in placental $PGF_{2\alpha}$ metabolism. Administration of this drug on days 9 and 10 resulted in a 68% decrease in $PGF_{2\alpha}$ metabolism; thus L10503 is a more potent inhibitor of $PGF_{2\alpha}$ metabolism than PGE_1 metabolism. Not only did this compound affect PG metabolism but it also reduced placental weight when administered on days 8, 9 and 10 of pregnancy, the greatest effect resulting from

Fig 1 Abortifacient activity of single subcutaneous injections of L10503 (a, c) or L10492 (b, d) on various days during the first 11 d of pregnancy in rats and hamsters. a: ———. 25 mg kg^{-1}; – – – –, 6.25 mg kg^{-1}; – . – . – ., 1.56 mg kg^{-1}. b: ———, 25 mg kg^{-1}; – – – –, 12.5 mg kg^{-1}. c and d: ———, 100 mg kg^{-1}; – – – –, 50 mg kg^{-1}.
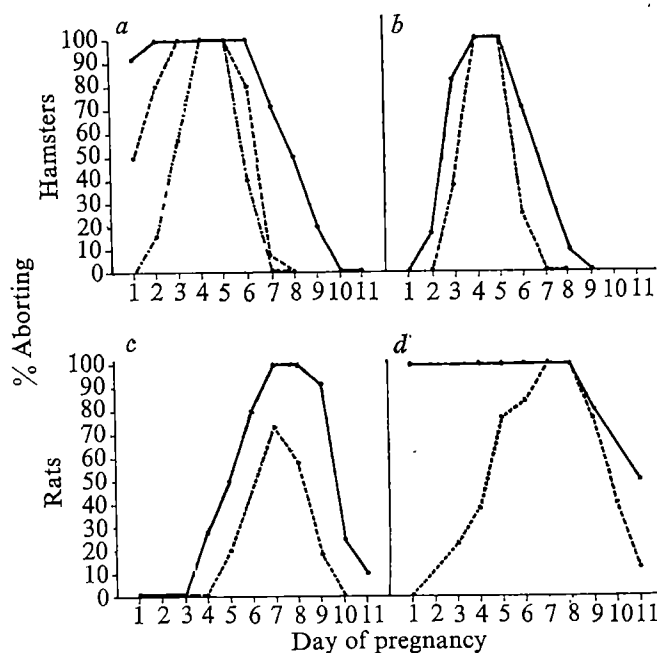
Table 2 Effect of L10503 treatment on various days of pregnancy on rat placental weight, prostaglandin metabolism and synthesis

Pregnancy day of L10503 treatment	Placental weight (mg)	PGE_1 pmol metabolised per h		$PGF_{2\alpha}$ pmol metabolised per h	
		per mg protein	per organ	per mg protein	per organ
8	88.5±8.1†	140.2±9.4	855.5±57.3*	7.5±1.2*	47.3±7.4*
9	88.7±5.1†	144.2±9.6	894.3±59.7*	8.2±1.3*	52 4±8.1*
10	94.2±5.0	125.7±10.3	817.4±66.8*	14 6±3.1	99.5±21.3†
9+10	73.2±1.4*	78.5±4.1*‡	392.5±20.6*§	5.9±0.7*	31.2±3.9*§
Sesame oil (9+10)	112.7±4.7	152.0±8.1	1185.6±63.0	18.2±0.6	149.6±5.3

		PGE_2 pmol synthesised per h		$PGF_{2\alpha}$ pmol synthesised per h	
		per mg protein	per organ	per mg protein	per organ
9	85.7±3.7	7.50±1.51	60.8±12.5	1.90±0.17	15.4±1.4
Sesame oil (9)	113.6±5.1	8.73±1.19	95.0±12.9	1.73±0.12	18.8±1.3

Values are mean ±s.e. of three pools of placentae from 4–8 rats each. Placentae preserved at −30 °C, homogenised in Bucher medium, 1:5 (w/v) for $PGF_{2\alpha}$ metabolism and 1:10 (w/v) for PGE_1 metabolism studies, and centrifuged at 10,000g for 15 min. To 2 ml aliquots of the supernatant were added 4 mM NAD^+, 0.1 μM $PGF_{2\alpha}$ (2.25 Ci $mmol^{-1}$) or 0.1 μM PGE_1 (2.45 Ci $mmol^{-1}$). Incubation time at 37 °C was 20 min for $PGF_{2\alpha}$ and 5 min for PGE_1 metabolism. Enzymatic reaction was stopped by addition of 0.2 ml 1 N HCl to acidify at pH 3.0. Extraction and separation by thin-layer chromatography of prostaglandins and their metabolites (15-keto and 15-keto-dihydro derivatives) were performed as described previously[15]. For PG synthesis studies, tissues were homogenised with 0.1 M Tris-HCl buffer pH 8.2, 1:5 (w/v). To 2 ml aliquots were added 4 mM adrenaline, 4 mM glutathione, and 1 μM ^{3}H-arachidonic acid (0.53 Ci $mmol^{-1}$). Time of incubation was 1 h and reaction was stopped by addition of citric acid to acidify at pH 3.0. Extraction and separation by thin-layer chromatography was as described previously[16].

Significance between treated and control groups was determined using Dunnett's t test; an F test following analysis of variance was used to compare data between experimental groups. *$P < 0.01$ and †$P < 0.05$ against vehicle control group; ‡$P < 0.01$ against day 9 treatment group; §$P < 0.01$ against day 10 treatment group.

treatment on both days 9 and 10. Note that even though placental weight was reduced by treatment, the concentration of protein per 100 mg tissue was unaffected (6.94±0.13 mg, mean ±s.e.). PG metabolism was therefore computed on the basis of total placental weight (Table 2). Treatment on any of the three pregnancy days studied resulted in significant reductions in PGE_1 metabolism. L10503 administered on days 9 and 10 decreased PGE_1 metabolism by 67%, and an even greater suppression of $PGF_{2\alpha}$ metabolism was observed after single day treatments on each of the three days of pregnancy. Administration of the compound on days 9 and 10 resulted in a 79% reduction in $PGF_{2\alpha}$ metabolism. *In vitro* studies also showed that L10503 inhibited $PGF_{2\alpha}$ metabolism although PGE_1 metabolism was unaffected.

PGE_2 and $PGF_{2\alpha}$ synthesis in the placenta was not significantly altered when L10503 (100 mg kg^{-1} subcutaneously) was administered on day 9 of pregnancy. PGE_2 synthesis in this tissue was four to five times greater than $PGF_{2\alpha}$ synthesis.

These studies on PG metabolism indicate that the mechanism of anti-fertility action of L10503 may involve the inhibition of placental PG metabolism, leading to an elevated level of endogenous PG which could be detrimental to placental physiology, that is, altered haemodynamics.

The toxicity (LD_{50}) of L10492 and L10503 is very low compared with the abortifacient doses (ED_{100}) when the compounds were administered for 5 consecutive days. The toxic dose was greater than 64 times and >320 times more than the ED_{100} doses for rat and hamster, respectively, in the case of L10492, and >8 times and >640 times more than the ED_{100} doses for rat and hamster, respectively, in the case of L10503.

High but not abortifacient doses of these anti-fertility agents have not produced teratogenic effects. Animals whose pregnancies were terminated with these drugs promptly returned to normal oestrous cycles, mated, became pregnant, had normal litters and lactated normally. The offspring resulting from these pregnancies were fertile at maturity.

These studies indicate that L10492 and L10503 are safe and effective anti-fertility agents that may produce their effect by inhibiting endogenous PG metabolism in the placenta. Furthermore these compounds may be useful in elucidating the role of PGs and their metabolism in placental function.

LEONARD J. LERNER
GIULIO GALLIANI
PAOLO CARMINATI
MARIA CARLA MOSCA

Lepetit Research Laboratories,
Gruppo Lepetit SpA,
Via Durando 38,
20158 Milano, Italy

1 Burdick, H. O., Whitney, R., and Pincus, G., *Anat. Record*, 67, 513 (1937).
2 Greenwald, G. S., *J. exp. Zool*, 135, 461 (1957).
3 Morris, J. M., and VanWagenen, G., *Am. J. Obst. Gynec.*, 96, 804 (1966).
4 Harper, M. J. K., *Endocrinology*, 81, 1091 (1967)
5 Naqvi, R H , and Warren, J. C., *Steroids*, 18, 731 (1971).
6 Lerner, L. J., in *Contraception· The Chemical Control of Fertility* (edit by Lednicer, D.), 161 (Dekker, New York, 1969).
7 Morris, J. M , VanWagenen, G , McCann, T., and Jacob, D , *Fert. Steril*, 18, 18 (1967).
8 Labsetwar, A. P , *Fedn. Proc* , 33, 61 (1974).
9 Pharris, B. B., and Behrman, M R., *The Prostaglandins*, 1, (edit by Tamwell, P W.), 347–363 (Plenum, New York, 1973).
10 Anderson, G G , and Speroff, L , *The Prostaglandins*, 1, (edit by Tamwell, P W.), 365–389 (Plenum, New York, 1973).
11 Duncan, G W., and Pharris, B. B , *Fedn Proc.*, 29, 1232 (1970).
12 Alam, N A , Clary, P., and Russell, P. T , *Prostaglandins*, 4, 363 (1973).
13 Craft, I. L , Scrivener, R., and Dewhurst, C. J., *J. Obstet. Gynaec. Br Commonw* , 80, 616 (1973)
14 Novy, M J , Piasecki, G., and Jackson, B. T , *Prostaglandins*, 5, 543 (1974).
15 Carminati, P., Luzzani, F., and Lerner, L J , *Prostaglandins*, 8, 205 (1974)
16 Flower, R. J., Cheung, H S., and Cushman, D. W., *Prostaglandins*, 4, 325 (1973).
17 Lerner, L. J , Galliani, G., Mosca, M C , and Omodei-Salé, A., *Fedn Proc.*, 34, 338 (1975).

Non-antiviral activities of interferon are not controlled by chromosome 21

USING mouse–human cell hybrids, Tan *et al.*[1] assigned the gene(s) for the expression of the antiviral state induced by human (leukocyte) interferon to chromosome 21; furthermore, human skin fibroblasts trisomic for chromosome 21 proved more sensitive to the antiviral activity of human (leukocyte) interferon than normal diploid fibroblasts or trisomic 18 or 13 fibroblasts[2,3]. Since the non-antiviral and antiviral activities of interferon remain inseparably linked through approximately a million-fold purification (ref. 4 and references cited therein), the question may be raised

whether these various activities of interferon are accounted for by the same mechanisms and whether the expression of the non-antiviral activity of (human) interferon is also controlled by chromosome 21.

We report here that—in comparison with normal diploid (D) cells, or trisomic 18 (T-18) or trisomic 13 (T-13) cells—trisomic 21 (T-21) cells are indeed more sensitive to the antiviral action but not to the non-antiviral effects of interferon. As parameters of the non-antiviral activity of interferon we chose 'priming' (increased responsiveness of interferon-treated cells to interferon induction by poly(I)·poly(C)[5,6]) and 'toxicity-enhancement' (increased susceptibility of interferon-treated cells to the cytotoxicity of double-stranded RNAs[7,8], such as poly(I)·poly(C)). Both the antiviral and the non-antiviral activities of interferon were explored with human leukocyte and fibroblast inter-

Fig. 2 Toxicity-enhancing activity of human leukocyte (*a*) and fibroblast (*b*) interferon in normal diploid fibroblasts (NS, JH) and fibroblasts trisomic for chromosome 13, 18 or 21. Semi-confluent cell cultures in 60 mm Falcon Petri dishes were treated with interferon at the indicated concentrations (in Eagle's MEM + 3% foetal calf serum: 2 ml per Petri dish) for 16 h at 37 °C, drained, washed, and then exposed to 100 µg ml⁻¹ of poly(I)·poly(C) (P-L Biochemicals, Milwaukee, Wisconsin) (in Eagle's MEM, 1 ml per Petri dish) for 1 h at 37°C. The plates were then washed and further incubated with serum-free Eagle's MEM (4 ml per Petri dish) for 16 h, at which time toxicity was monitored by neutral red uptake[7]. Symbols represent mean values for three experiments. ●, NS; ▲, JH; ○, T-13; △, T-18; □, T-21.

Fig. 1 Antiviral activity of human leukocyte (*a*) and fibroblast (*b*) interferon in normal diploid fibroblasts (two cell lines, designated NS and JH, respectively) and fibroblast trisomic for chromosome 13, 18 or 21. Wells of a semi-micro Linbro plate were seeded with 10^5 cells ml⁻¹ (Eagle's MEM + 10% foetal calf serum) per well. When confluent (usually after 4 d), the cells were maintained for another 3 d in EMEM + 3% foetal calf serum. The cultures were then treated with our standard leukocyte or fibroblast interferon preparations, diluted (in EMEM + 3% foetal calf serum) to give approximately 1.5 $\log_{10}$ units ml⁻¹ in NS cells, for 16 h at 37 °C, drained, washed, challenged with Mengo virus and further processed by the neutral red uptake technique as described by Finter[27]. The amounts of stain incorporated were determined by optical density readings at 542 nm. The readings made for the virus controls did not differ significantly from one cell type to another; nor did the readings made for the different cell controls. Hence, gross differences in normal cell metabolism or virus replication pattern between the different cell types employed may be excluded. Bars show means (+ s.d.) for three experiments.

feron preparations. Our studies indicate that, unlike the antiviral activity, the non-antiviral effects of human leukocyte and fibroblast interferon are not genetically determined, or at least not by chromosome 21.

Human leukocyte and fibroblast interferon were prepared according to well-established procedures[9,10]. The leukocyte and fibroblast interferon preparations employed had specific activities of approximately 10^4 units per mg protein. Interferon titres are expressed in terms of the British Research Standard B for human interferon (69/19). Fibroblast cultures were established from skin biopsies. The cells were subcultured in Eagle's minimal essential medium (MEM) supplemented with 10% foetal calf serum. All cell cultures were matched as nearly as possible with regard to the number of cell generations in culture.

In agreement with Tan's findings[2,3], our experiments showed that T-21 cells were about ten times more sensitive

to the antiviral action of human leukocyte intereferon than either T-13 or T-18 or normal diploid cells (Fig. 1). In addition, T-21 cells were more sensitive than T-13, T-18 or diploid cells to the antiviral action of human fibroblast interferon (Fig. 1).

The inhibitory effect of poly(I)·poly(C) on virus multiplication is thought to be mediated by interferon induction even at polymer concentrations which do not lead to the release of detectable amounts of interferon in the cell culture medium[11-13]. If this assumption is correct, one may expect poly(I)·poly(C) to be more active in inhibiting virus replication in T-21 than in normal diploid cells. As shown before[2], poly(I)·poly(C) was indeed more active in T-21 than in normal diploid cells: the minimal concentration of poly(I)·poly(C) required to suppress cytopathogenicity induced by vesicular stomatitis virus was 0.003 µg ml⁻¹ in T-21 cells but 0.03 µg ml⁻¹ in three normal diploid cell lines (designated NS, JH and FS-4).

In accord with previous findings[7], human fibroblast interferon enhanced the susceptibility of human fibroblasts to the toxicity of poly(I)·poly(C) (Fig. 2). The extent of cell destruction, as monitored by neutral red uptake, closely paralleled the amounts of interferon applied. Leukocyte interferon proved equally effective as fibroblast interferon in enhancing the toxicity of poly(I)·poly(C). Most importantly, all cell lines tested, whether disomic or trisomic, behaved quite similarly in their sensitivity to the toxicity-enhancing activity of interferon (Fig. 2).

Similar results were obtained with regard to the priming activity of interferon (Fig. 3) : no marked differences were noted in the sensitivities of T-13, T-18, T-21 and normal diploid cells to the priming effect of either human leukocyte or fibroblast interferon.

Two major conclusions follow from these results. First, though the antiviral activity of human leukocyte and fibroblast interferon seems to be controlled by gene(s) located on chromosome 21, their non-antiviral activities are not. It is even arguable whether the non-antiviral activities of interferon are genetically regulated at all. On several occasions it has been suggested, albeit without direct experimental evidence, that the priming as well as the toxicity-enhancing effect of interferon are solely cell surface (or
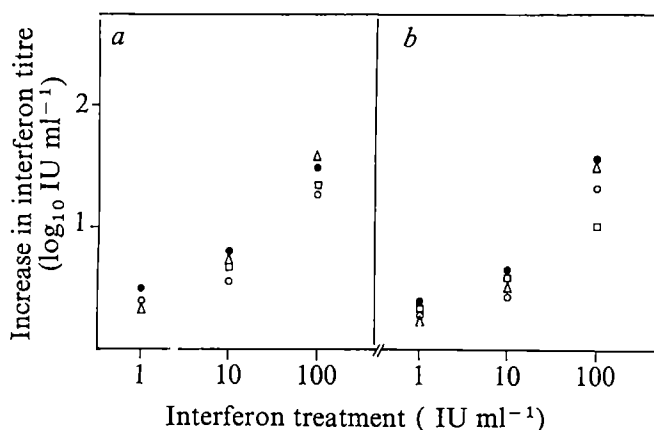
Fig. 3 Priming activity of human leukocyte (*a*) and fibroblast (*b*) interferon in normal diploid fibroblasts (NS) and fibroblasts trisomic for chromosome 13, 18 or 21. The procedure followed for measuring the priming activity of interferon diverged from that applied for measuring the toxicity-enhancing activity (legend to Fig. 2) in that confluent (instead of semiconfluent) cell cultures were used (about 5×10^6 cells per Petri dish), poly(I)·poly(C) was added at 50 (instead of 100) μg ml^{-1}, and the plates were incubated with Eagle's MEM+3% foetal calf serum (instead of serum-free medium) after their exposure to poly(I)·poly(C). The supernatant fluids collected 16 h after poly(I)·poly(C) had been removed were assayed for interferon by the dye uptake method[27] in human skin fibroblasts (NS) challenged with Mengo virus. The interferon titres are expressed as the differences in titre obtained between the 'primed' and control cell cultures. ●, NS; ○, T-13; △, T-18; □, T-21.

cell membrane) phenomena[8,14,15]. The data presented here and previous data[5,8], eliminating the necessity of protein synthesis for the expression of the priming and toxicity-enhancing effects of interferon, would seem consistent with this hypothesis. Although the data reported here indicate fundamental differences in the mechanisms underlying the antiviral and non-antiviral activities of interferon, they do not rule out the possibility that these mechanisms only diverge from a certain point onward: for example both the antiviral and non-antiviral effects of interferon may be triggered from the cell surface[16–18]. Whereas the 'priming' and toxicity enhancement could be envisaged as the direct consequence of an alteration of the cell membrane, the expression of the antiviral state would require a few additional steps involving derepression, transcription and, finally, translation of the putative antiviral protein.

Second, human leukocyte interferon and human fibroblast interferon differ considerably in their biological properties (antiviral activity in heterologous cells[19–21]), physicochemical properties (stability towards heat[22], shaking[23], guanidine treatment[23], reactivation by anionic detergents[24]) and antigenic properties (as assessed by neutralisation with specific antisera[25,26]). These differences may imply that human leukocyte interferon and human fibroblast interferon differ structurally[25] and/or contain distinct molecular species[24], and, if our interferon preparations contain different molecular species, the question arises whether the antiviral and non-antiviral activities of the interferon preparation reside in one and the same or in separate molecular species. Yet, in spite of these differences, leukocyte and fibroblast interferon exhibited similar antiviral, priming and toxicity-enhancing activities in the fibroblast cell lines studied. Hence, human leukocyte interferon and human fibroblast interferon should share a common structural determinant that initiates both the antiviral and non-antiviral responses (possibly from a common receptor site at the cell surface, as suggested above).

We thank Mrs A. Van Lierde, Mrs M. Stuyck, Miss I. Ronsse, Miss M. Scheys and Mrs G. Koninckx for technical

ERIK DE CLERCQ
VICTOR G. EDY
JEAN-JACQUES CASSIMAN

*Rega Institute for Medical Research
and Division of Human Genetics,
Department of Human Biology,
University of Leuven,
Leuven, Belgium*

1 Tan, Y. H., Tischfield, J., and Ruddle, F. H., *J. exp. Med.*, 137, 317–330 (1973).
2 Tan, Y. H., Schneider, E. L., Tischfield, J., Epstein, C. J., and Ruddle, F. H., *Science*, 186, 61–63 (1974).
3 Tan, Y. H., *Nature*, 253, 280–282 (1975).
4 Stewart, W. E, II, *et al.*, *Nature new Biol*, 246, 141–143 (1973)
5 Stewart, W. E., II, Gosser, L B., and Lockart, R. Z, Jr, *J. Virol.*, 7, 792–801 (1971)
6 De Clercq, E., Stewart, W E., II, and De Somer, P., *Infect. Immun*, 8, 309–316 (1973).
7 Stewart, W. E, II, De Clercq, E., Billiau, A, Desmyter, J, and De Somer, P., *Proc. natn. Acad Sci. U.S A.*, 69, 1851–1854 (1972)
8 Stewart, W. E., II, De Clercq, E., and De Somer, P. *J. gen. Virol.*, 18, 237–246 (1973).
9 Strander, H., Cantell, K., Carlstrom, G., and Jakobsson, P. A., *J natn. Cancer Inst.*, 51, 733–742 (1973).
10 Billiau, A., Joniau, M., and De Somer, P, *J gen. Virol*, 19, 1–8 (1973)
11 Schäfer, T W, and Lockart, R. Z., Jr, *Nature*, 226, 449–450 (1970).
12 De Clercq, E., and Merigan, T. C, *J. gen. Virol.*, 10, 125–130 (1971)
13 Vilcek, J., and Varacalli, F., *J gen. Virol*, 13, 185–188 (1971).
14 Kleinschmidt, W. J., *A. Rev Biochem*, 41, 517–542 (1972).
15 De Clercq, E, and De Somer, P., *J gen. Virol.*, 27, 35–44 (1975).
16 Ankel, H., Chany, C., Galliot, B, Chevalier, M. J., and Robert, M., *Proc. natn. Acad Sci. U.S.A.*, 70, 2360–2363 (1973)
17 Knight, E., Jr, *Biochem. biophys. Res. Commun.*, 56, 860–864 (1974).
18 Knight, E., Jr, *J gen. Virol*, 25, 143–145 (1974)
19 Desmyter, J, Rawls, W. E., and Melnick, J. L., *Ann N Y. Acad. Sci.*, 173, 492–504 (1970).
20 Levy-Koenig, R. E., Golgher, R. R., and Paucker, K., *J. Immun.*, 104, 791–797 (1970).
21 Gresser, I., Bandu, M.-T., Brouty-Boyé, D., and Tovey, M, *Nature*, 251, 543–545 (1974).
22 Mogensen, K. E., and Cantell, K., *Acta path. microbiol scand*, 81B, 382–383 (1973).
23 Edy, V. G., Billiau, A., Joniau, M., and De Somer, P., *Proc. Soc exp Biol. Med.* 146, 249–253 (1974).
24 Stewart, W. E., II, *et al*, *J. gen. Virol.*, 26, 327–331 (1975).
25 Anfinsen, C. B, Bose, S., Corley, L., and Gurari-Rotman, D., *Proc. natn Acad. Sci. U S.A.*, 71, 3139–3142 (1974)
26 Berg, K, Ogburn, C. A., Paucker, K., Mogensen, K E, and Cantell, K., *J. Immun.*, 114, 640–644 (1975).
27 Finter, B. N., *J gen. Virol.*, 5, 419–427 (1969).

Saturable, energy-dependent, transmembrane transport of prostaglandins against concentration gradients

SOME vertebrate tissues, such as the choroid plexus, anterior uvea, and the kidney cortex, accumulate radioactivity against a concentration gradient when incubated with ^{3}H-prostaglandins (^{3}H-PGs)[1]. This accumulation is reversible, temperature-dependent and can be inhibited by metabolic and transport inhibitors, and by high concentrations of PGs, PG analogues and some organic acids such as probenecid and indomethacin[2,3].

It has been argued[1] that *in vitro* accumulation of PGs by the choroid plexus and anterior uvea reflects the transmembrane transport of these biologically active fatty acids across the blood–brain and blood–ocular fluid barriers. Such active PG absorption from the extracellular fluids of brain and eye could have considerable physiological significance as these potentially harmful autocoids are produced, but not effectively metabolised, by intraocular[4] and central nervous tissues[5]. Recent experiments have shown that rabbit erythrocytes are impermeable to PGs of the E and F series, indicating that cellular membranes can represent effective barriers to the free diffusion of these autocoids[6]. We must consider therefore that the elimination of PGs from the extracellular fluids of eye and brain, the intracellular metabolism of PGs by the lung, and their excretion by the kidneys require specific transmembrane transport processes[7].

In vivo experiments have already provided evidence that

PGs are transported out of the intraocular[8] and cerebrospinal[9] fluids and are absorbed from the vaginal lumen[10] of rabbits by facilitated, carrier-mediated mechanisms. None of these *in vivo* experiments, however, could provide direct evidence for active transport of chemically unaltered PGs as the rapid metabolism of PGs by lung and liver prevents the chemical identification of the transported PGs and the maintenance of constant PG concentration in the blood. Whereas *in vitro* studies demonstrated that PG uptake against a concentration gradient is not primarily associated with a chemical alteration of the PG
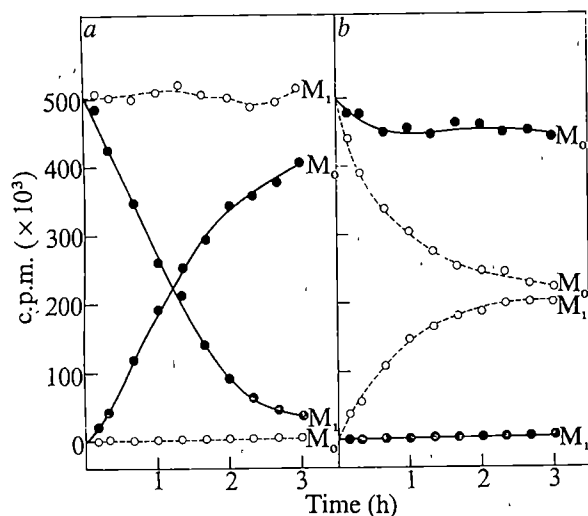
Fig. 1 Unidirectional ^3H and ^{14}C fluxes across isolated rabbit vagina. *a*, M_i to M_o fluxes. Initially M_i contained ^3H-PGF$_{2\alpha}$ (●) plus PGF$_{2\alpha}$ (total [PGF$_{2\alpha}$] = $10^{-6}M$) plus ^{14}C-sucrose (○), whereas M_o contained no PG or radioactivity. Rapid outward movement of ^3H activity reversed initial ^3H gradient within 90 min. *b*, M_o to M_i fluxes. Initially M_i contained no radioactivity and no PG whereas M_o contained ^3H-PGF$_{2\alpha}$ (as above; ●) plus ^{14}C-thiourea (○). Entry of ^3H-PGF$_{2\alpha}$ into the tissue resulted in some ^3H loss from M_o, but there was no evidence of ^3H-PGF$_{2\alpha}$ entry into the lumen. In contrast, ^{14}C-thiourea, a substance which freely penetrates cell membranes, rapidly crossed the tissue and a thiourea equilibrium between M_o and M_i was approached within 2 h.

New Zealand white rabbits (2.1–3 3 kg) were killed with an overdose of Nembutal. The vagina was freed by blunt dissection and cut near the pelvic bone and just below the cervix. One end of the resulting tube was fitted with, and tied to, a Plexiglass tube and the other end was ligated to form a bladder-like preparation. The resulting sac was filled with 1 or 2 ml Earle's tissue culture medium (Microbiological Associates) and was lowered into a vessel containing 2 ml culture medium. The Plexiglass tube served as a reservoir for the inside medium during the periods of vaginal constrictions which typically lasted for 10 s with a periodicity of 0.5 to 3 min To minimise variations in results, only vaginas which could be fitted over the 8 mm diameter tip of the Plexiglass tube and the walls of which were translucent, and less than about 2 mm thick in the relaxed state, were used. As the surface areas of these bladder-like preparations could not be accurately determined, individual rates could not be standardised to a unit surface area. Each figure represents, therefore, results obtained on one preparation, typical of a set of four or more similar experiments The assembly was maintained in a bath at 37 °C or at 2–4 °C. In most experiments, trace amounts of ^{14}C-sucrose, ^{14}C-thiourea, ^{14}C-octanoic acid or ^{14}C-linoleic acid were also added to either M_o, M_i, or both, to test the passive permeability properties of the vagina and the specificity of this transport system Samples (20 µl) of both M_o and M_i were taken at 5–20 min intervals, oxidised using a Packard Tricarb Model 306 automatic sample oxidiser, and the separate ^3H and ^{14}C samples counted for 10 min or 10,000 counts in a liquid scintillation counter. In some experiments both media were changed, after 60 or 90 min, to one containing a high concentration of unlabelled PG, or another potential inhibitor, in addition to the ^3H and ^{14}C-labelled compounds After 30–50 min of further sampling, both inside and outside compartments were washed with, and replaced by, a medium containing a higher inhibitor concentration or with the original, inhibitor-free, radioactive medium.

molecule[11], such uptake studies could not distinguish between PG adsorption on to specific binding sites and active transport of free PGs into interstitial fluid compartments.

In the present experiments, a bladder-like preparation of the isolated rabbit vagina was used as a model system to demonstrate that chemically unaltered PG can indeed be transported across a biological membrane against a chemical concentration gradient by a temperature and energy-dependent, saturable process.

Either the inside (M_i) or the outside (M_o) or both media contained ^3H-PGF$_{2\alpha}$ or ^3H-PGE$_1$ (New England Nuclear) and both sides were aerated with 95 % O_2, 5 % CO_2.

In experiments in which initially only M_i contained ^{14}C-sucrose and ^3H-PGF$_{2\alpha}$, there was a rapid entry of ^3H into M_o, but there was no significant loss of ^{14}C from M_i or detectable entry of ^{14}C into M_o. The results of a typical experiment are shown in Fig. 1a. In four identical experiments, the observed $M_i \rightarrow M_o$ ^3H transfer corresponded to a total apparent PGF$_{2\alpha}$ flux of 400 ng h^{-1}. Thus, the rabbit vagina, presumably its epithelial lining, allows a rapid flux of PGF$_{2\alpha}$ in the outward direction, yet it is a permeability barrier to sucrose, a substance of essentially identical molecular weight.

When initially only M_o contained ^3H-PGF$_{2\alpha}$, there was an initial decrease in ^3H activity of M_o in all five such experiments, presumably due to the entry of ^3H-PGF$_{2\alpha}$ into the tissue itself, but there was no detectable entry of ^3H activity into M_i. The result of a typical experiment of this series is shown in Fig. 1b. Thus, the rabbit vagina is an effective barrier to the influx of PGF$_{2\alpha}$ into the lumen while allowing a large PGF$_{2\alpha}$ efflux from the lumen to the outside. This outward ^3H flux could not be caused by passive ^3H-PGF$_{2\alpha}$ diffusion only, as after the first 90 min of incubation, the ^3H concentration in M_o surpassed that of M_i (Fig. 1a).

When the vagina was inverted before insertion of the cannula, the direction of the net ^3H flux remained 'mucosal' →'serosal'. Experiments with ^{14}C-linoleic or ^{14}C-octanoic acid showed no evidence of transmembrane transport of these unrelated fatty acids.

When initially both M_o and M_i contained the same ^3H-PGF$_{2\alpha}$ concentration, the ^3H activity of M_i steadily decreased, whereas that of M_o showed a continuous increase between 10 and 60 min of incubation. This resulted in the establishment of a continually increasing ^3H gradient across the isolated vagina reaching a more than 18-fold gradient by 2 h incubation at 37 °C (Fig. 2a). The mean ^3H gradient in five such experiments was 5.2 ± 0.8 ($n = 13$) at the end of 1 h, reaching a peak of 14 ± 4 ($n = 4$) at 3 h incubation at 37 °C. Similar results were obtained when ^3H-PGE$_1$ was used instead of ^3H-PGF$_{2\alpha}$, but the extent of the maximum ^3H gradient was lower (three to sevenfold between 2 and 4 h incubation).

At 4 °C, the ^3H activity of M_o remained equal to M_i until the temperature was raised to 37 °C (Fig. 2a). When both media contained 2 mM iodoacetate, and N_2 in the aeration mixture, net flux of ^3H was not observed, and M_o/M_i remained at unity indefinitely at 37 °C (Fig. 2a). The $M_i \rightarrow M_o$ flux was partially inhibited by 10^{-5} M, and was almost completely blocked by 10^{-4} M PGF$_{2\alpha}$ (Fig. 2b). PGF$_{2\beta}$, a relatively inactive analogue of PGF$_{2\alpha}$ (ref. 12) was found to be more effective than PGF$_{2\alpha}$ itself, yielding essentially complete inhibition of ^3H-PGF$_{2\alpha}$ transport at 10^{-6} M. The unrelated fatty acid, octanoic acid, produced partial inhibition only at a concentration of 10^{-3} M.

To determine how much of the ^3H activity transported across the vagina remained associated with the original PG, both M_o and M_i were removed after 90 or 120 min of incubation and extracted for PGs (ref. 13). Paper chromatography[14] of such extracts showed that only about two-thirds of the ^3H activity of M_o remained associated with PGF$_{2\alpha}$, whereas approximately one-fourth of the ^3H was found with the 15-keto-PGF$_{2\alpha}$ spot. For this reason 10^{-4} .M furosemide was used in all further experiments to inhibit the 15-dehydrogenase activity[15]. In the presence of furosemide the ^3H gradient
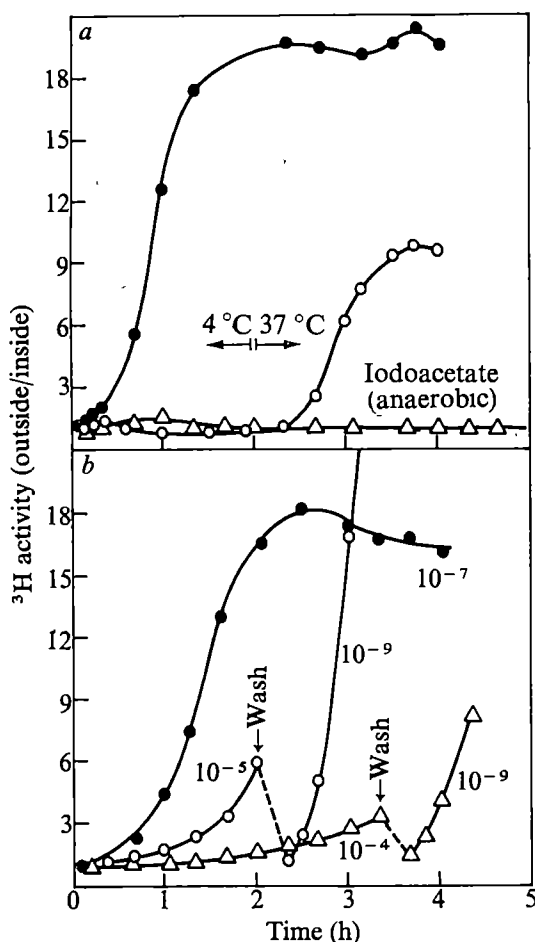
Fig. 2 Effects of temperature, metabolic inhibition and high $PGF_{2\alpha}$ concentrations on development of ^3H gradient across isolated rabbit vagina incubated with ^3H-$PGF_{2\alpha}$. *a*, When M_i and M_o had the same initial ^3H-$PGF_{2\alpha}$ activity (total $[PGF_{2\alpha}]$ approximately 10^{-9} M) the $M_i \rightarrow M_o$ flux established a 20-fold M_i/M_o ^3H gradient at 37 °C (●), whereas at 4 °C the M_i/M_o ratio remained at unity until the temperature was raised to 37 °C (O). The ^3H ratio remained at unity indefinitely in anaerobic conditions (at 37 °C) in the presence of 2 mM iodoacetate (△). *b*, The development of M_o/M_i ^3H gradient showed a dose-dependent, reversible inhibition when the $[PGF_{2\alpha}]$ in both M_o and M_i was raised from 10^{-7} (●) to 10^{-5} (O) or 10^{-4} M (△).

was slightly reduced, but the formation of 15-keto-$PGF_{2\alpha}$ was greatly suppressed; thus, in five experiments $89 \pm 2\%$ ^3H activity in M_o and $91 \pm 1\%$ in M_i remained associated with the $PGF_{2\alpha}$ spot on the A1 system[14]. In this condition, a net flux of chromatographically identifiable ^3H-$PGF_{2\alpha}$ against a concentration gradient was observed which resulted in a final, paper chromatographically identifiable, $PGF_{2\alpha}$ M_o/M_i ratio of 7.3 ± 2.5 ($n = 5$). The fact that in the presence of furosemide the bulk of the accumulated ^3H remained associated with $PGF_{2\alpha}$ was also substantiated in four such experiments with a previously described[11] immunoadsorption technique.

To provide further evidence that $PGF_{2\alpha}$ transport does not require a metabolic alteration of the PG molecule, five similar experiments were carried out in which both M_i and M_o contained 10^{-6} M $PGF_{2\alpha}$ and 10^{-4} M furosemide but no ^3H-$PGF_{2\alpha}$. After 2 h incubation, the fluids were removed and the PGs extracted[13]. Immunoassay of these extracts with an anti-$PGF_{2\alpha}$ serum which shows negligible cross-reactivity with 15-keto-$PGF_{2\alpha}$ (ref. 11), showed that the $PGF_{2\alpha}$ content of M_i decreased, whereas that of M_o increased, yielding more than fivefold final M_o/M_i $PGF_{2\alpha}$ gradient. Bioassay of three of these extracts on gerbil colon[16] also showed that the $PGF_{2\alpha}$-like activity of M_o increased whereas that of M_i decreased. As in all these experiments the initial $PGF_{2\alpha}$ concentration of

$M_i = M_o$, these results substantiate the isotope studies indicating that chemically unaltered, biologically active, $PGF_{2\alpha}$ can be transported across a biological membrane against a true concentration gradient.

In three similar experiments, $PGF_{2\alpha}$ was omitted from the initial incubation medium to see whether locally synthesised $PGF_{2\alpha}$ is released into M_o or M_i. After 2 h incubation, a small amount (0.6 ± 0.1 ng; $n = 3$) of $PGF_{2\alpha}$ was found by immunoassay in M_o only. This amount of PG release could not significantly affect the observed PG gradient, but indicates that PGs which are synthesised by the vagina are either released entirely on the serosal side of the barrier or are transported across this barrier preventing their accumulation in the lumen.

The existence of such active PG transport processes could have physiological significance, not only in the case of the vagina[10], blood-ocular fluid[8] and blood-brain [9]barriers, but also with respect to the general distribution and metabolism of these autocoids[7]. PG accumulation against a concentration gradient by the kidney cortex[1], for example, suggests that excretion and/or renal distribution of PGs and/or their metabolites also depends on a carrier-mediated process. PG accumulation by lung tissue[1] and its saturable uptake into isolated perfused rat lung preparation[17] suggests that a carrier-mediated uptake of PGs into the cells may be required for the rapid pulmonary metabolism of PGs.

In conclusion, the energy-dependent, saturable net flux of $PGF_{2\alpha}$ across the isolated rabbit vagina provides direct experimental evidence in support of the concept[1] that PGs can be actively transported by some vertebrate tissues. Concurrent *in vitro* studies on rabbit anterior uvea, choroid plexus and kidney cortex[2,3] indicate that this PG transport mechanism is distinctly different from the iodide transport system, it overlaps with the p-aminohippurate system and may be related to the 'L' component of the iodipamide transport system which was described recently[18]. This PG transport system can be expected to have a fundamental role in the removal of PGs from the extracellular fluids of the eye[8] and the brain[9] and may also have a role in the pulmonary metabolism and renal excretion of PG (ref. 7). The existence of such PG transport processes implies that inhibitors of PG transport may provide an experimental and/or therapeutic tool for the manipulation of the distribution and disposition of PGs.

Laszlo Z. Bito

Department of Ophthalmology,
College of Physicians and Surgeons,
Columbia University,
New York, New York 10032

1 Bito, L. Z., *J. Physiol*, *Lond*, 221, 371–387 (1972), *Comp Biochem. Physiol*, 43A, 65–82 (1972)
2 Bito, L. Z, *Fedn Proc*, 33, 589 (1974).
3 Bito, L Z, Davson, H, and Salvador, E V., *J Physiol., Lond* (in the press)
4 Eakins, K E, Atwal, M, and Bhattacherjee, P, *Expl Eye Res*, 19, 141–146 (1974)
5 Nakano, J., Prancan, A. V, and Moore, S. E., *Brain Res*, 39, 545–548 (1972)
6 Bito, L. Z, and Baroody, R, *Fedn Proc*. 34, 791 (1975), *Am J. Physiol* (in the press)
7 Bito, L Z, *Prostaglandins* (in the press)
8 Bito, L Z, and Salvador, E. V., *Expl Eye Res.*, 14, 233–241 (1972)
9 Bito, L Z, and Davson, H, *J. Physiol*, *Lond*, 236, 39P–40P (1973)
10 Bito, L. Z, and Spellane, P J, *Prostaglandins*. 8, 345–352 (1974).
11 Bito, L Z, and Baroody, R, *Prostaglandins*, 7, 131–140 (1974).
12 Bundy, G L., Yankee, E V, Weeks, J R, and Miller, W. L, *Adv Biosci*, 9, 125–133 (1973)
13 Unger, W. G., Stamford, I F, and Bennett, A, *Nature*, 233, 336–337 (1971)
14 Stamford, I F, and Unger, W G, *J Physiol*, *Lond*, 225, 4P–5P (1972).
15 Paulsrud, J R, and Miller, O. N, *Fedn Proc*, 33, 590 (1974).
16 Eakins, K E., Whitelocke R. A. F., Perkins, E S, Bennett, A, and Unger, W. G., *Nature new Biol*, 239, 248–249 (1972)
17 Anderson, M. W., *Fedn Proc*, 34, 790 (1975)
18 Barany, E H, *Acta physiol. scand.*, 88, 412–429 (1973).

Differential effects of cyclic AMP on the *in vitro* induction of antibody synthesis

INTRACELLULAR events in lymphocytes following antigenic stimulation still remain largely obscure. It has recently been demonstrated that cyclic AMP is involved during early events of the *in vitro* immune induction, since exogenous application of cyclic AMP can prevent spleen lymphocytes from differentiating into antibody-producing cells[1]. One can assume that this is more than a general effect on cell division processes, since inhibition by cyclic AMP could be demonstrated only during the first 24 h of the immune induction period, whereas cell proliferation starts after 24 h (ref. 2). Since cooperation between T and B cells and proliferation as well as differentiation of these cells are essential features during the early period of the immune response against most antigens[3] it was of interest to study the effect of cyclic AMP on the primary immune response in a T-cell-deficient system such as nude spleen cell cultures. Nude spleen cells can be stimulated to antibody production against sheep red blood cells (SRBC) not only in the presence of allogeneic T cells and antigen[4,5] but also by B cell mitogens, such as lipopolysaccharides (LPS) from various bacteria without a specific antigen[6].

Here, the effect of exogenous cyclic AMP on the T-cell–dependent immune response to SRBC is compared with the T-cell–independent activation by the mitogen LPS and possible events following treatment by cyclic AMP are discussed.

As shown in Table 1, nude spleen cells give rise to only small numbers of antibody-producing cells in the absence of allogeneic thymus cells or a potent mitogen like LPS.

and specific antigen (Table 1), whereas cultures reconstituted with thymus lymphocytes and stimulated by SRBC showed a reduction in the number of plaque-forming cells on day 4.

It was of interest to see whether cyclic AMP affects those events which occur during the cooperation of thymus-derived and bone marrow-derived lymphocytes or rather other processes which are involved in antigen recognition and T cell maturation. Therefore, antigen-activated T cells, as matured T cells, were used to restore the immunological capacity of nude spleen cells in place of allogeneic thymus cells. The effect of cyclic AMP in this culture system was tested and compared with cultures restored with normal allogeneic thymocytes from 4-week-old donors.

From the data of Table 2 we can see that the induction of antibody synthesis is suppressed by cyclic AMP only when normal allogeneic thymus lymphocytes are used for restoration. The addition of cyclic AMP to nude spleen cells restored with antigen-activated T cells did not lead to inhibition of day 4 antibody synthesis against SRBC. No difference in the insusceptibility to cyclic AMP was seen when donors of three different H-2 backgrounds were used as the source of activated T cells. This cooperation across the H_2-barrier may suggest parallels to allogeneic stimulation. In this case, one must conclude that the cyclic AMP independence of antigen-activated T cells in this respect indicates that they have reached a stage in development differing from normal allogeneic T cells.

Differences in the intracellular level of cyclic AMP have been described for different stages in differentiation of mammalian cells[10]. Since it was found that inhibition of the immune induction by cyclic AMP can be effected only during the first 24 h (ref. 1), it was of interest to see whether this intracellular mediator is involved in cooperation events during the early period of the immune induction or rather influences processes

Table 1 Influence of cyclic AMP on the immune response to SRBC in *nu/nu* spleen cells

| Cell cultures | PFC per 10⁶ collected cells | | | | |
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
nu/nu spleen cells (3 d)	79	—	60	68	55
nu/nu spleen cells (3 d)+SRBC	33	—	72	86	94
nu/nu spleen cells (3 d)+LPS	184	172	310	390	213
nu/nu spleen cells (3 d)+LPS+SRBC	203	142	—	—	—
nu/nu spleen cells (3 d)+LPS+cyclic AMP	164	231	309	295	264
nu/nu spleen cells (4 d)+SRBC	47	63	—	—	—
nu/nu spleen cells (4 d)+SRBC+Thy	303	299	—	—	—
nu/nu spleen cells (4 d)+SRBC+Thy +cyclic AMP	56	54	—	—	—

Cell cultures of spleen cells from *nu/nu* mice with NMRI background were prepared according to the method of Mishell and Dutton[7]. Sheep erythrocytes (SRBC) were used as antigen at a dose of $3 \times 10^6 - 5 \times 10^6$ SRBC per 10^7 cells. Culture dishes reconstituted with thymus lymphocytes contained 1×10^7 nude spleen cells and 5×10^6 thymus cells or 5×10^6 antigen-activated thymus cells, whereas cell cultures supplemented with LPS contained only 1.5×10^7 nude spleen cells. After 3 or 4 d of *in vitro* incubation five culture dishes of one group were pooled. The number of antibody-producing cells was determined with a modified Jerne plaque technique[8]. Cell viability was checked by Trypan blue dye exclusion tests. The number of plaque-forming cells was determined after two different times of incubation *in vitro*. Spleen cells stimulated with LPS were collected on day 3 and cell cultures restored by allogeneic spleen cells were pooled and assayed on day 4 of incubation. Lipopolysaccharides (LPS) from *Salmonella anatum* were used to stimulate nude spleen cell cultures. LPS was extracted by the phenol–water method and purified by ultracentrifugation[9]. It was dissolved in Eagle's medium to a final concentration of 10 μg ml⁻¹. Thy, allogeneic thymus lymphocytes from 4-week-old C57BL; cyclic AMP was used at 10^{-3} mol.

On day 3 the number of plaque-forming cells in cultures to which SRBC had been added did not differ significantly from control cultures without antigen. On the other hand when LPS or allogeneic thymus lymphocytes were added, these nude spleen cell cultures produced increased numbers of plaque-forming cells on day 3 or day 4. There was no significant difference in the 3-d response between cultures supplemented with LPS alone or with both LPS and SRBC, when 10 μg ml⁻¹ LPS was used.

Parallel cultures were treated with 10^{-3} mol cyclic AMP (Boehringer, Mannheim) at the beginning of incubation. This dose was shown to inhibit the immune induction of normal spleen cultures in previous experiments[1]. The application of cyclic AMP has no inhibitory effect on nude spleen cell cultures activated polyclonally with LPS in the absence of thymus cells

dependent on antigenic interaction with T cells.

The data presented here lead to the conclusion that exogenous application of cyclic AMP probably affects those events in the immune induction which are related to T cell function, since LPS-induced differentiation of B cells to antibody-producing cells cannot be disturbed by exogenous cyclic AMP. Also experiments with antigen-activated T cells indicate that the interaction of T and B cells itself is not affected by cyclic AMP, since cooperation can occur in nude cell cultures restored by matured T cells in the presence of cyclic AMP. Because only one of the three culture conditions presented here was influenced by cyclic AMP (namely, nude spleen cells restored by naive thymus lymphocytes) one can conclude that exogenous cyclic AMP disturbs processes which lead to T cell maturation after antigenic stimulation.

Table 2 Influence of cyclic AMP on the immune response to SRBC in *nu/nu* spleen cells* restored with antigen-activated T cells

Cell cultures		PFC per 10^6 collected cells	
	Experiment 1†	Experiment 2‡	Experiment 3§
nu/nu spleen cells + SRBC	5	0	13
nu/nu spleen cells + Thy + SRBC	161	281	157
nu/nu spleen cells + t-act + SRBC	195	489	309
nu/nu spleen cells + Thy + SRBC + cyclic AMP	35	2	2
nu/nu spleen cells + t-act + SRBC + cyclic AMP	408	680	300

For the preparation of antigen-activated T cells 8–12-week-old mice were lethally irradiated with 750 r. They received $1 \times 10^7 - 3 \times 10^7$ thymus lymphocytes intravenously from a syngeneic donor, 4 weeks old. At the same time 3×10^8 SRBC were injected intraperitoneally into each recipient. After 6 d mice were killed, their spleen cells collected and used as antigen-activated T cells (t-act)

Thy, thymus lymphocytes from 4-week-old C57BL; PFC, plaque forming cells. -

* *nu/nu* mice derived from the 10th backcross generation with BALB/c.

† C57 mice ⎫

‡ C3H mice ⎬ donors of antigen-activated T cells.

§ BALB/c mice ⎭

The differential effect of cyclic AMP on nude spleen cell cultures supplemented either with allogeneic thymus cells or antigen-activated T cells corresponds to the differential effect of cyclic AMP in normal spleen cultures, when the immune response is prevented only during a critical time during immune induction[1]. Therefore it seems reasonable to relate the action of this cyclic nucleotide in normal cells to early processes in thymus lymphocytes induced by antigen. Although Watson *et al.* found that the immune response is inhibited when B cells are treated with cyclic AMP before antigen presentation[12], more recent results of Watson[13] and others (J. Anderson, F. Melchers, and S. Bauminger, personal communication) demonstrate that the intracellular level of cyclic AMP in nude spleen cells is not changed significantly after stimulation by LPS, whereas the intracellular level of cyclic GMP rises dramatically.

Our results are also related to other reports on cyclic AMP binding sites on the cell surface of thymus lymphocytes and intracellular measurements which show changed levels of cyclic AMP in thymus lymphocytes[11].

Treatment of nude spleen cells with theophylline results in inhibition of antibody production after LPS stimulation (R. Bösing-Schneider, unpublished). This effect is not understood, because the intracellular level of cyclic AMP in these cells is not changed after stimulation by LPS (ref. 13). Since cyclic GMP seems to have an important role in these cells[13] one can question if the cyclic GMP phosphodiesterase is affected by theophylline.

Our experiments have shown that exogenous cyclic AMP had an inhibitory influence only in cell cultures restored by allogeneic thymocytes and stimulated by antigen, whereas in cultures activated polyclonally[6] with LPS, in the absence of thymus cells and antigen, no inhibition effect was detectable. Further experiments with antigen-activated T cells lead to the conclusion that treatment of cell cultures with cyclic AMP may affect only those antigen dependent processes which lead to T cell maturation.

RITA BÖSING-SCHNEIDER

Immunology Unit,
Biology Division,
University Konstanz,
775 Konstanz, West Germany

1 Bosing-Schneider, R., and Kolb, H., *Nature*, 244, 224–225 (1973)
2 Dutton, R. W., and Mishell, R. J., *J. exp. Med*, 126, 443–454 (1967)
3 Miller, J. F A. P., and Mitchell, G. F., *J exp Med.*, 128, 801–837 (1968)
4 Schimpl, A, and Wecker, E, *Eur. J. Immun.*, 1, 304–306 (1971).
5 Bosing-Schneider, R., and Kindred, B, *Cell Immun.*, 4, 593–596 (1972)
6 Andersson, J., Sjoberg, O, and Moller, G, *Europ. J. Immun*, 2, 349–353 (1972).
7 Mishell, R J., and Dutton, R. W, *J. exp. Med.*, 126, 423–442 (1967).
8 Weiler, E., Melletz, E. W., and Breuninger-Peck, E, *Proc. natn. Acad. Sci. U.S.A.*, 54, 1310–1317 (1965).
9 Westphal, O, and Jann, K., *Methods in Carbohydrate Chemistry*, 5, 83–91 (1965).
10 *Advances in Cyclic Nucleotide Research* (edit. by Greengard, P., and Robinson, G. A.), 4 (Raven, New York, 1974).
11 *Cyclic AMP, Cell Growth, and the Immune Response* (edit. by Braun, W., Lichtenstein, L M, and Parker, C W.), (Springer, Berlin-Heidelberg-New York 1974).
12 Watson, J, Epstein, R., and Cohn, M, *Nature*, 246, 405 (1973).
13 Watson, J., *J. exp Med.*, 141, 97–111 (1975).

Evidence for the induction of two antibodies with identical combining sites in outbred animals

THE mechanism by which the immune system produces an apparently limitless array of antibodies in response to a variety of antigenic stimuli remains an unresolved biological question. Primary structural analysis has shown that immunoglobulin heavy (H) and light (L) chains have both variable (V) and constant (C) regions of amino acid sequence. Within the V_H and V_L region sequences there are areas of hypervariability which are thought to be associated with the antibody combining site. It is the amino acid substitutions within certain of these hypervariable areas that result in the multiplicity of antibody specificities (sites) expressed in nature. It seems clear that separate genes code for the V and C regions of H and L chains and that integration of V- and C-region genes occurs at the DNA level[1]. One can account for antibody isotypic diversity by postulating a limited number of C-region genes which are transmitted from generation to generation in the germ line. The difficulty arises in the attempt to account for the apparent large numbers of V-region genes required to explain antibody combining site diversity. V-gene counting has been accomplished by RNA–DNA hybridisation[2-4]. Evidence from these studies has not definitely demonstrated whether the capacity to generate large numbers of combining sites exclusively arises by either the inheritance of a complete set of V_H and V_L genes in the germ line or by somatic mutation. The repeated occurrence of V-gene products has, however, been indicated by sequence analysis of myeloma proteins[5-7], isoelectric focusing[8-10], and idiotypic analysis[11-13].

It thus seems that an abundance of evidence favours repeated V-region genes in inbred systems and supports the germ line hypothesis[14-16]. It now becomes important to map particular V regions and to show whether they are stably retained in an outbred population. Support for the stability of V-region genes in outbred systems comes from the discovery of apparently identical phosphorylcholine combining sites in different inbred mouse strains[13] as well as highly similar V-region sequences (differing by only 8 residues, 3 being located in the hypervariable regions) in two human IgM myeloma heavy chains obtained from genetically distinct individuals[17]. To gain additional insight into V-region gene transmission and stability in a randomly outbred species we are currently mapping V regions from antibodies directed against the DNP determinant. Our studies have taken advantage of the antigen (DNP)$_2$-gramicidin S,

which reproducibly elicits antibodies of limited structural heterogeneity (refs 18, 19), thereby maximising recognition of particular V regions. Here we examine evidence suggesting that antibodies with identical combining sites have been elicited in two outbred rabbits and provide additional evidence that the genetic information necessary to account for V-region (combining site) diversity is present and stably transmitted in the germ line.

A detailed analysis of the immune response to $(DNP)_2$-gramicidin S has been reported previously[18,19]. These studies showed that a number of rabbits producing anti-DNP antibodies possessed monoclonal characteristics[19]. Initial assessment of monoclonality was based on: (1) the spectral similarities of these antibodies to myeloma proteins and (2) the lack of binding heterogeneity as reflected in unitary heterogeneity indices. Further evidence for homogeneity was obtained from chain reassociation studies in which these purified antibodies regained their original binding constants after reduction, alkylation and exposure to dissociating conditions[19].

Figure 1a shows the isolectric spectra of six rabbits possessing monoclonal spectrotypes. It is evident that the monoclonal responses were not confined to rabbits possessing identical serum allotypes nor did individual antibodies necessarily possess identical spectrotypes. Two antibodies

Fig. 1 Serum aliquots (100 μl) or purified antibodies (200–400 μg) were focused in 5% thin-layer polyacrylamide gels containing pH 3.5–10 or 6–8 ampholine carrier ampholytes (LKB). Anti-DNP antibodies were seen after interaction with ^{131}I HDL, by contact autoradiography[18]. a, Isoelectric analysis of serum from rabbit 13 (track 1, allotype a13, b44), rabbit 22 (track 2, allotype a12, b44), rabbit 71 (track 3, allotype a13, b44), rabbit 126 (track 4, allotype a33, b44), rabbit 127 (track 5, allotype a33, b44) and rabbit 129 (track 6, allotype a13, b44) on pH 3.5–10 ampholyte gels. These characteristic monoclonal anti-DNP antibody spectra were perpetuated with antigenic stimulation until the death of each rabbit. b, Isoelectric analysis of antibodies from rabbits 13 (tracks 5 and 6; 6 and 18 months after initial immunisation) and 22 (1, 2 and 3; 6, 12 and 24 months after initial immunisation) on a pH 6–8 ampholyte gel. Track 4 was obtained by mixing equal quantities of purified antibodies from each rabbit.
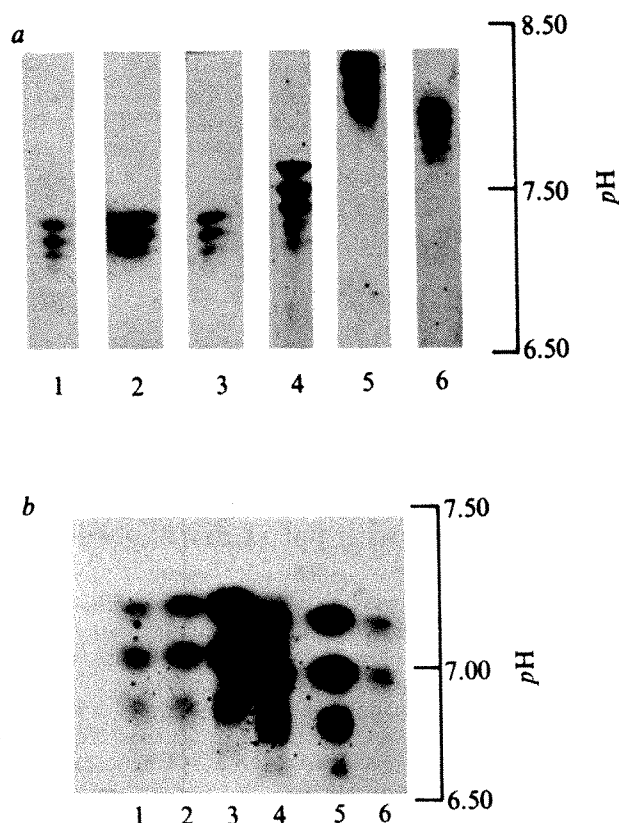
Table 1 Association constants of anti-DNP antibodies before and after recombination*

Sample	Mean K_0 (M^{-1})	
	Before	After
MOPC-315	1.2×10^6	1.3×10^6
		(1.2×10^6)†
anti-DNP–BGG (pool)	4.6×10^9	6.7×10^5
		(7.6×10^5)†
anti-DNP–GS		
13	1.3×10^6	(1.2×10^6)†
22	1.4×10^6	(1.2×10^6)†
H 13‡	$< 10^5$	—
L 13‡	NA§	—
H 22	$< 10^5$	—
L 22	NA§	—
H13‡+L22	—	1.2×10^6
H22 +L13‡	—	1.3×10^6
H22 +L (Ab pool)‖	—	$< 10^5$

*Recoveries of 7S recombinants compared with the starting material ranged from 30 to 55%, the dialysis bag method averaging 5% higher recovery than the chain separation method. Recombinant recoveries for pooled anti-DNP–BGG antibodies and heterologous chains [H22+L (Ab pool)] ranged from 30–35%. Recombinant recoveries for MOPC-315, 13, 22, and 13 and 22 cross chain mixtures were comparable and ranged from 50 to 55%.
†( ) experiments using non-separated chains; dialysis bag method.
‡[14]C-labelled chains.
§No measurable activity.
‖Light chains obtained from pool of heterogeneous anti-DNP antibodies.

(track 1, rabbit 13, and track 2, rabbit 22) which seemed to display similar spectrotypes, were analysed further on isoelectric gels containing expanded range ampholytes. Figure 1b shows a detailed analysis of rabbits 22 (tracks 1, 2, 3,) and 13 (tracks 5 and 6). Track 4 was obtained by mixing equal quantities of these two antibodies and shows that the spectra are not, in fact, identical. In view of our subsequent findings, these slight spectral differences may result from biochemical modifications outside the combining site and perhaps outside the V region. In spite of spectral differences, both antibodies possess identical association constants (Table 1) which suggests possible combining site similarities.

To test this possibility we undertook a series of chain recombination experiments. Chain recombination has been used previously to assess the heterogeneity of mouse[20] and rabbit[21] antibodies. Our preliminary recombination studies with rabbit 13 and 22 antibodies had shown that complete regain of binding activity could be obtained when chains from homologous antibodies were allowed to reassociate Table 1)[19], a behaviour characteristic of monoclonal antibodies[20,21]. We used cross chain recombination studies to study the relationship of the combining site structure of these two antibodies. Fig. 2 outlines the experimental protocols used in this analysis. The quantities of monoclonal antibodies precluded the exclusive use of separated chains in every experiment, so initially it was important to compare the two recombination protocols. Table 1 summarises the data from these experiments. The K_0 values on the left were obtained before reduction and alkylation, the values on the right after reassociation following the indicated protocol. Mouse MOPC-315 IgA, a myeloma protein with reactivity for DNP, completely regained its original K_0, whereas a heterogeneous population of rabbit anti-DNP antibodies showed a drop of 4 log units. These data confirmed that the recombination system can distinguish monoclonal and heterogeneous antibody populations and justified the use of either recombination protocol. The data on the two monoclonal antibodies show that recombinant 7S molecules prepared from either H (13) and L (22) or H (22) and L (13) displayed K_0 values identical to recombinants prepared with homologous H and L chains. Control experiments showed that H (13) or H (22) did not possess K_0 values of parent molecules, nor did recombinants prepared using these H chains and L chains from other anti-

PURIFIED 7S ANTIBODY-K_0

Mild reduction and alkylation

Chain separation

Exposure acid

Mixing of H and L

Recombination:

Chromatography:

K_0

Dialysis to neutral pH

7S recombinant antibody

(K_0)
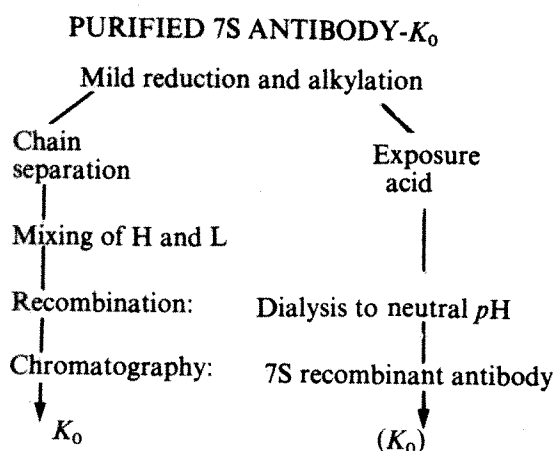
Fig. 2 Alternative protocols used in the recombination studies. Antibodies were purified by affinity chromatography on DNP–Sepharose immunoadsorbent columns[19] and aggregates removed by chromatography & (Sephadex G-150). 7S antibodies were mildly reduced for 2 h in 0.1 M 2-mercaptoethanol in 0.2 M Tris (pH 8.0) under N_2 at 25 °C followed by alkylation for 30 min with a 10% molar excess of iodoacetamide (in certain cases 1-^{14}C iodoacetamide was used to label the H and L chains). The dissociation of the mildly reduced and alkylated chains was either accomplished by dialysis against 1 M propionic acid or by physical separation on Sephadex G-100 equilibrated in the same solvent. Separated chains (mixed in a ratio of 1.2 mol L chain per mol H chain) or dissociated chains were allowed to reassociate by sequential dialysis against 500-fold excess amounts of 0.05 M propionic acid (once), H_2O (twice) and borate buffered saline (pH 8.3) (twice). Recombinant 7S molecules were recovered from a calibrated Sephadex G-150 column[19]. Association constants (K_0) were determined by fluorescence quenching at 4 °C (ref. 19).Each K_0 value represents the average of at least three titrations.

DNP antibodies (Table 1 and unpublished observations). Jaton (Basel Institute for Immunology) has carried out recombination studies[22] on homogeneous anti-pneumococcal type III antibodies which have been sequenced[23,29]. Recombinant molecules prepared with H chains and heterologous L chains differing by as few as 6 hypervariable residues (total of 8 substitutions within the amino terminal 139 residues) do not regain appreciable binding activity, whereas recombinants prepared with homologous H and L chains do[22]. In fact, to date, Jaton (personal communication) and others[25] have only been able to achieve the binding activity of the parent molecule when H and L chains come from the same parent molecule. Our argument for combining site identity would, of course, be strengthened by amino acid sequence data. Jaton's findings demonstrate, however, that the chain recombination technique is a powerful probe of combining site fine structure[22]. The interpretation of our data in the light of these findings leads to the suggestion that rabbits 13 and 22 most probably possess identical combining sites.

The probability of two such antibodies arising purely by somatic mutations and being expressed in 2 out of as few as 100 rabbits seems low. Confidence in the random breeding of these two rabbits comes from the knowledge that each was immunised in a different country. The stability of these responses is illustrated by the fact that they were potentiated up to 1.5 (rabbit 13) to 2 (rabbit 22) years, without variation. When stimulation was discontinued for 6 months (rabbit 13), the response disappeared. Restimulation induced identical monoclonal antibody. Recent studies have suggested that two or more genes may code for heavy chain V regions, one specifying framework residues including the group a allotype, another specifying the idiotype[26,27]. The two rabbits studied in detail here (rabbits 13 and 22) were heterozygous for the a allotype, both sharing the

a1 allotype. Finding the same allotype on antibodies with identical combining sites would suggest repetition of the entire V region, although sequence data would be needed to confirm such a speculation. It remains possible that a given combining site is not confined to a particular allotype and present investigations are directed at determining if identical sites may be present on antibodies of different allotypes.

The apparent ability to induce identical combining sites in outbred members of the same species lends strong additional support to the notion that a particular V-region gene may persist and be transmitted stably in the germ line while apparently undergoing minimal mutations in regions not affecting the combining site.

We thank Dr R. Mage for her generosity in carrying out the allotype determinations, Dr J.-C. Jaton for making his data available before publication, and Dr A. Williamson for critical comments on the manuscript.

P. C. MONTGOMERY
C. A. SKANDERA
R. L. KAHN

*Department of Microbiology and Center for Oral Health Research,
University of Pennsylvania School of Dental Medicine,
Philadelphia, Pennsylvania 19174*

1 Milstein, C., and Munro, A. J., *Defense and Recognition, Biochemistry Series One* (edit. by Porter, R. R.), **10**, 199–228 (University Park Press, Baltimore, 1973).
2 Premkumar, E., Shoyab, M., and Williamson, A. R., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 99–103 (1974).
3 Leder, P., Honjo, T., Packman, S., Swan, D., Nav, M., and Norman, B., in *The Immune System Genes, Receptors and Signals* (edit. by Sercarz, E. E., Williamson, A. R., and Fox, C. F.), 299–317 (Academic, New York, 1974).
4 Tonegawa, S., Steinberg, C., Dube, S., and Bernardini, A., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4027–4031 (1974).
5 Weigert, M. G., Cesari, I. M., Yonkovich, S. J., and Cohn, M., *Nature*, **228**, 1045–1047 (1970).
6 Cohn, M., Blomberg, B., Geckeler, W., Raschke, W., Riblet, R., and Weigert, M., *The Immune System, Genes, Receptors and Signals* (edit. by Sercarz, E. E., Williamson, A. R., and Fox, C. F.), 99–117 (Academic, New York, 1974).
7 Barstad, P., Farnsworth, V., Weigert, M., Cohn, M., and Hood, L., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4096–4100 (1974).
8 Kreth, H. W., and Williamson, A. R., *Eur. J. Immun.*, **3**, 141–147 (1973).
9 Schlossman, S. F., and Williamson, A. R., *Genetic Control of Immune Responsiveness* (edit. by McDevitt, H. O., and Landy, M.), 54–60 (Academic, New York, 1972).
10 Pink, J. R. L., and Askonas, B. A., *Eur. J. Immun.*, **4**, 426–430 (1974).
11 Eichmann, K., *Eur. J. Immun.*, **2**, 301–307 (1972).
12 Kindt, T. J., Seide, R. K., Bokisch, V. A., and Krause, R. M., *J. exp. Med.*, **138**, 522–536 (1973).
13 Claflin, J. L., and Davie, J. M., *J. exp. Med.*, **140**, 673–686 (1974).
14 Hood, L., and Talmage, D. W., *Science*, **168**, 325–334 (1970).
15 Wigzell, H., *Scand. J. Immun.*, **2**, 199–206 (1973).
16 Hood, L., Barstad, P., Loh, E., and Nottenburg, C., *The Immune System, Genes, Receptors and Signals* (edit. by Sercarz, E. E., Williamson, A. R., and Fox, C. F.), 119–139 (Academic Press, New York, 1974).
17 Capra, J. D., and Kehoe, J. M., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4032–4036 (1974).
18 Montgomery, P. C., Rockey, J. H., and Williamson, A. R., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 228–232 (1972).
19 Montgomery, P. C., Rockey, J. H., Kahn R. L., and Skandera, C. A., *J. Immun.* (in the press).
20 Klinman, N. R., *J. Immun.*, **106**, 1330–1337 (1971).
21 Ghose, A. C., and Karush, F., *J. Immun.*, **113**, 162–172 (1974).
22 Huser, H., Haimovich, J., and Jaton, J-C., *Eur. J. Immun.*, **5**, 206–210 (1975).
23 Jaton, J-C., *Biochem. J.*, **141**, 1–13 (1974).
24 Jaton, J-C., *Biochem. J.*, **141**, 15–25 (1974).
25 Bridges, S. H., and Little, J. R., *Biochemistry*, **10**, 2525–2530 (1971).
26 Kindt, T. J., Thunberg, A. L., Mudgett, M., and Klapper, D. G., *The Immune System, Genes, Receptors and Signals* (edit. by Sercarz, E. E., Williamson, A. R., and Fox, C. F.), 69–98 (Academic, New York, 1974).
27 Capra, J. D., and Kindt, T. J., *Immunogenetics*, **1**, 417–427 (1975).

Detection of reverse transcriptase in human breast tumours with poly(Cm)·oligo(dG)

PARTICLES having the density of type-C RNA tumour viruses, possessing reverse transcriptase (RNA-directed DNA polymerase) activity, and containing high molecular weight RNA that shares a portion of its base sequences with the RNA of murine RNA tumour viruses, have been reported to be present in several types of human malignant tissues, but not in their normal counterparts (review, see ref. 1). The endogenous RNA→DNA synthetic capabilities of these particles were used in a simultaneous detection test[2,3] to determine these parameters. Because of their

similarity and relatedness to animal oncornaviruses, these particles are considered to be possible aetiological agents of human cancer[2].

To establish more firmly the presence of particles containing reverse transcriptase activity in human tumours, and to delineate the difficulties involved in the use of an exogenous synthetic probe to detect the enzyme in crude extracts, we have examined a number of human tissues for reverse transcriptase activity using poly(2'-o-methylcytidylate)·oligodeoxyguanylate [poly(Cm)·oligo(dG)] as template primer. Poly(Cm)·oligo(dG) is an effective template primer for every reverse transcriptase so far tested, including the enzymes from RNA tumour viruses of avian, murine, feline, and primate origin[4] (G.F.G., unpublished). Neither bacterial DNA polymerase I nor any of the eukaryotic DNA polymerases tested, including the three major DNA polymerase species from mouse 3T6 or human KB and HeLa cells[1,4,5], can copy poly(Cm)·oligo(dG). In particular, highly purified DNA polymerase γ from HeLa cells, an enzyme that resembles viral reverse transcriptase in its ability to copy poly(C)·oligo(dG)[6], does not copy poly(Cm)·oligo(dG)[5]. Poly(Cm)·oligo(dG) is therefore a specific template primer for viral reverse transcriptase. Using this specific probe, we have detected reverse transcriptase activity in particulate material isolated from the postmitochondrial cytoplasm of approximately 50% of the

Fig. 1 Identification of the density of reverse transcriptase and phosphatase activity in a postmitochondrial extract of human mammary tumour by sucrose gradient centrifugation. A P-90 pellet from 13 g of human mammary tumour was prepared and fractionated on a 15–65% sucrose gradient as described in the legend to Table 1. Individual fractions within nine density regions were pooled, particulate material pelleted, and the pellets suspended as in Table 1. Samples (10 μl) from each suspension were diluted and assayed for dGTP phosphatase (open bars) as follows. The phosphatase sample was added to a reaction mixture (50 μl) containing 20 mM Tris-HCl (pH 7.8), 0.2 mM MnCl$_2$, and 3.7 μM ³H-dGTP (2,900 c.p.m. pmol^{-1}). After 30 min at 37 °C, the reaction was terminated by heating at 100 °C for 2 min. The amount of ³H-dGTP remaining in the reaction mixture was then determined by using it as a substrate for excess DNA polymerase. A solution (50 μl) was added to the heated mixture containing 10 mM Tris-HCl (pH 7.8), 10 mM dithiothreitol, 5 mM MgCl$_2$, 0.1 mM each dCTP, dATP, and dTTP, 300 μg ml^{-1} activated calf thymus DNA, and 1.4 units of *Micrococcus luteus* DNA polymerase (P-L Biochemicals). After a 60 min incubation at 37 °C, the amount of labelled DNA product formed was determined on Whatman DE-81 filters[4]. The total remaining suspensions (990 μl) from density regions III (1.185–1.170 g cm^{-3}), IV (1.165–1.150 g cm^{-3}), V (1.145–1.130 g cm^{-3}), and VI (1.125–1.115 g cm^{-3}), were lysed, fractionated on separate phosphocellulose columns, and assayed for reverse transcriptase (solid bars) and transferase as in the legend to Table 1. Density regions I and II were directly assayed for reverse transcriptase activity after lysis without fractionation on phosphocellulose. No activity was observed in I and II. No transferase activity was observed in any density region.

human mammary tumours we have examined, but not in the cytoplasm of non-malignant breast tissue. It was necessary to remove interfering deoxyribonucleotide phosphatase activity present in human tissue extracts by phosphocellulose chromatography before reverse transcriptase activity could be detected.

We have taken the approach of isolating particulate material with a density between 1.15 and 1.20 g cm^{-3}, a density range characteristic of RNA tumour viruses, from the postmitochondrial cytoplasm of human tissue, and after treating the particulate material with non-ionic detergent, assaying for DNA polymerase activity with poly(Cm)·oligo (dG) (for experimental details, see the legend to Table 1). This approach minimises intereference in the assay by cellular enzymes, such as nucleases and other polymerases[2,3]. It was discovered, however, that all tissue extracts examined, regardless of the human organ of origin, contained an inhibitor of poly(Cm)·oligo(dG)-directed poly(dG) synthesis, deoxyribonucleotide phosphatase (Fig. 1). By paper chromatographic analysis, the phosphatase activity was found to hydrolyse ³H-dGTP or ³H-TTP through mono and diphosphate intermediates to the deoxyribonucleoside level (G.F.G., unpublished). To facilitate removal of phosphatase from the cytoplasmic particulate fraction of tissues, we developed a rapid and convenient assay for dGTP phosphatase that uses residual ³H-dGTP in an activated calf thymus DNA-directed polymerising reaction catalysed by excess DNA polymerase (G.F.G. and P.M.L., in preparation). Details are described in the legend to Fig. 1.

Using this assay, we found that the postmitochondrial, cytoplasmic particulate fraction from 10 g of human liver, spleen, kidney, or breast that banded in the 1.15 to 1.20 g cm^{-3} density region of 15–65% sucrose gradients, contained sufficient phosphatase to hydrolyse from 0.8 to 1.2 μmol of ³H-dGTP in 30 min. To carry out a poly(Cm)·oligo(dG)-directed assay in standard conditions without inhibition by phosphatase, the level of the enzyme in particulate cytoplasmic extracts would have to be reduced by 500 to 1,000-fold. The only technique we have found which removes enough phosphatase activity is phosphocellulose chromatography. In the presence of 0.1 M NaCl, phosphatase activity is not adsorbed by phosphocellulose, whereas reverse transcriptase from particles treated with Nonidet P-40 (NP-40) is bound and can be eluted with 0.25 M NaCl. By seeding human tissues (liver, kidney, spleen, breast) with various concentrations of RNA tumour viruses such as murine sarcoma-leukaemia or RD114 virus and using the procedure described in the legend to Table 1 to isolate reverse transcriptase, we have found in general that from 14 to 38% of the reverse transcriptase activity responding to poly(Cm)·oligo(dG) present in seeded virus is recovered in a volume of 2 ml from phosphocellulose columns.

Table 1 summarises our results with 19 human breast carcinomas and 9 non-malignant control tissues. Phosphocellulose eluates were assayed for DNA polymerase activity with poly(Cm)·oligo(dG), for terminal deoxyribonucleotidyl transferase activity with oligo(dG)[7], and for background radioactivity without template or primer. Backgrounds averaging 235 ± 72 c.p.m. were subtracted in all cases. No significant transferase activity was detected in any of the tissue samples examined. The average incorporation observed with the non-malignant control tissues with poly(Cm)·oligo(dG) was 59 c.p.m. In contrast, the average with the malignant tissues was 837 c.p.m. On the basis of the magnitude and variability of the background trapping of radioactivity, there was a 90% probability that all tissues which gave 200 c.p.m. or more incorporation above background were positive for activity. On this evaluation, 53% of the malignant samples were positive for reverse transcriptase by the poly(Cm)·oligo(dG) assay and all the control samples were negative. It should be pointed out that 5 of

the 9 malignant tissues which were negative weighed less than 5 g, which may have had a direct bearing on the low level of enzyme activity observed.

In addition to activity with poly(Cm)·oligo(dG), we have also examined phosphocellulose eluates prepared from a number of human carcinomas and non-malignant tissues for DNA polymerase activity with poly(C)·oligo(dG) and poly(A)·oligo(dT) in the presence of $MnCl_2$. Tissues negative for activity with poly(Cm)·oligo(dG) were also negative with poly(C)·oligo(dG), and positive responses to these template primers were comparable in magnitude. In contrast, there was not always a correlation between response to poly(Cm)·oligo(dG) and to poly(A)·oligo(dT). For example, in both normal and malignant breast tissue extracts in which there was no response to poly(Cm)·oligo(dG), we sometimes observed no activity with poly(A) oligo(dT) or activity which catalysed the incorporation of from 700 to 20,000 c.p.m. of ³H-TTP in response to poly(A)·oligo(dT). This activity probably represents variable contamination by DNA polymerase γ (ref. 6) of tissue extracts prepared by

Table 1 Detection of reverse transcriptase activity in breast tissue by poly(2'-o-methylcytidylate)·oligodeoxyguanylate

Type	Donor no.	Weight (g)	Poly(Cm) · oligo(dG)	Oligo(dG)	Designation
Breast tissue description			**Incorporation of ³H-dGTP** (c p m. above background per 60 min incubation) with		
Malignant					
Comedoadenocarcinoma	P-329	6.7	798 415* 747†	ND‡ ND	+
Carcinoma	P-284	10.8	4,065	83	+
Infiltrative ductal adenocarcinoma	R-277A	8.7	314	−29	+
Adenocarcinoma	R-295A	10.2	2,079 847*	16 ND	+
Infiltrative ductal adenocarcinoma	R-291A	13.8	1,493 732*	−22 −45	+
Carcinoma	P-286	5.0	3,124 2,846*	42 24	+
Infiltrative ductal carcinoma	R-307A	5.4	962	18	+
Carcinoma	P-101	7.3	220	122	+
Infiltrative ductal adenocarcinoma	P-230	5.8	185 208*	ND ND	+
Carcinoma	P-80	10	2,289 2,463*	ND ND	+
Carcinoma	D-686C	11.9	1	ND	−
Carcinoma simplex	B-213A	6.2	86	−28	−
Carcinoma	P-241	3.6	34	12	−
Papillary carcinoma	R-204B	2.0	−8	6	−
Comedocarcinoma	D-662A	3 3	47	−67	−
Infiltrative ductal carcinoma	R-202C	5.7	−9	0	−
Carcinoma	D-797A	9.7	68	32	−
Infiltrative ductal adenocarcinoma	P-486	3.8	85	95	−
Infiltrative ductal adenocarcinoma	P-670	4.3	74	54	−
Non-malignant					
Normal	74-368	11.8	0	10	−
Normal	74-353	11.5	−25	−37	−
Normal	SM-3	3.9	−34	23	−
Fibrocystic	SM-1	9.9	84	29	−
Fibrocystic	P-636	8.8	129	5	−
Fibrocystic	P-644	13.9	53	49	−
Fibrocystic	P-649	8.3	116	4	−
Fibrocystic	P-651	5.8	115	91	−
Fibromastitis	P-635	5.1	93	3	−

Frozen human breast tissue, 2–14g, was finely minced and disrupted with a Silverson homogeniser at 4 °C in 3 volumes of 0.01 M Tris-HCl (pH 8.3) +0.15 M NaCl+0.002 M EDTA (TNE)³ The suspension was centrifuged at 4,000g for 20 min at 4 °C and the pellet washed with 1 volume of TNE and recentrifuged The supernatants were combined and centrifuged again at 10,000g for 10 min. The pellet was washed with TNE and recentrifuged The 10,000g supernatants were combined and treated with trypsin (1 mg ml⁻¹ for 30 min at 37 °C) which was inactivated with Lima bean trypsin inhibitor (0.4 mg ml⁻¹). The treated supernatant was layered on a 12-ml column of 20% glycerol in TNE and spun at 90,000g for 1 h at 4 °C in an SW27 rotor. The resulting pellet (P-90) was suspended in 4 ml of TNE and layered on a 30 ml 15–65% (w/v) sucrose gradient in TNE and centrifuged at 90,000g for 12–14 h in an SW27 rotor Fractions (40 to 50) were collected from below, and those in the density range of 1.145 to 1.20 g cm⁻³ were combined, diluted with TNE to bring the sucrose concentration below 10%, and centrifuged at 150,000g for 1 h in a Ti60 rotor. The resulting pellet was suspended in 1 ml of 0.02 M Tris-HCl (pH 7.8)+0 001 M dithiothreitol+0 1 mM EDTA+ 10% glycerol (Buffer A), NaCl and NP-40 were added to 0.1 M and 2%, and the lysate incubated at 0 °C for 15 min. The lysate was applied to a 0.9×3 cm phosphocellulose (P-11, Whatman) column equilibrated in Buffer A +0.2% NP-40. The column was washed with 20 ml of Buffer A+0.2% NP-40+0.1 M NaCl, and eluted with 10 ml of Buffer A+0.2% NP-40+0.8 M NaCl. Fractions of 1 ml were collected. A sample (250 µl) of the first fraction from the phosphocellulose column containing an increased concentration of NaCl (usually 0.15 to 0.2 M) was incubated for 1 h at 37 °C in a reaction mixture (500 µl) containing 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 0.2 mM MnCl₂, 30 µM poly(Cm), 15 µM (dG)₁₂₋₁₈, and 2–4 µM ³H-dGTP (2 3–2.7×10⁴ c.p.m. pmol⁻¹). Additional samples were assayed (i) in the absence of poly(Cm) and (ii) in the absence of both poly (Cm) and (dG)₁₂₋₁₈. The amount of trichloracetic acid insoluble radioactivity in the reaction mixture was determined by collection of the product on nitrocellulose filters after washing by repeated precipitation and dissolution with perchloric acid and NaOH⁴.
* A repeat 1-h incubation was carried out.
† A repeat 2-h incubation was carried out.
‡ Not determined.
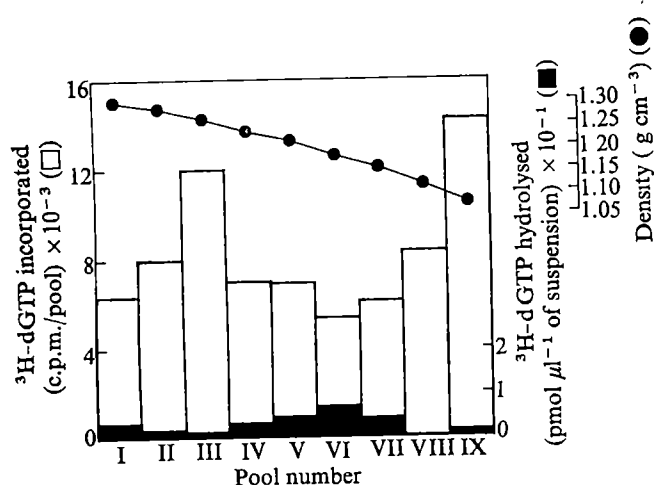
Fig. 2 Identification of the density of reverse transcriptase and phosphatase activity in an extract of human mammary tumour treated with nonionic detergent. A P-90 pellet from 50 g of human mammary tumour was prepared and fractionated on five, 15–65% sucrose gradients and particulate material with a density of 1.15 to 1.20 g cm⁻³ was pooled and pelleted in a Ti60 rotor as described in the legend to Table 1. The pelleted material was suspended in 600 µl of 0.1 M Tris-HCl (pH 8.3), and dithiothreitol and Sterox-SL were added to final concentrations of 100 mM and 1%, respectively. The lysate was layered on a 11.5-ml gradient of 25–85% (w/v) in TNE and was centrifuged at 180,000 g for 14 h in a SW41 rotor. The gradient was fractionated into nine density fractions, and the fractions were diluted with TNE to bring the sucrose concentration below 10% and were centrifuged at 150,000g for 1 h in a Ti60 rotor. The resulting pellets were suspended in 200 µl of 0.01 M Tris-HCl, 0.01 M dithiothreitol, and 0.5% NP-40. Samples (10 µl) from each lysate were assayed for phosphatase (solid bars) as described in the legend to Fig. 1. Samples (50 µl) were directly assayed for reverse transcriptase activity (open bars) and transferase activity in 100 µl reaction mixtures as in Table 1. The average background incorporation was 225 c.p.m., and no appreciable transferase activity was observed in any fraction.

our procedures. The relative template efficiencies of poly(C)·oligo(dG), poly(A)·oligo(dT) and poly(Cm)·oligo(dG) with MnCl₂ for a purified mammalian RNA tumour virus (murine leukaemia virus) reverse transcriptase are 1.0, 0.7 and 0.2, respectively (refs 4 and 5 and G.F.G., unpublished).

To demonstrate that the DNA polymerase activity responding to poly(Cm)·oligo(dG) observed in human mammary tumours, but not in non-malignant breast tissue, is derived from RNA tumour virus-like particles, the DNA polymerase-containing material was further characterised with respect to two properties. First, the exact density of the particulate, poly(Cm)·oligo(dG)-directed DNA polymerase activity from a human breast tumour was determined by sucrose gradient equilibrium centrifugation. Figure 1 shows that the majority of material which contained DNA polymerase activity responding to poly(Cm)·oligo(dG) banded between 1.15 and 1.165 g cm⁻³, a density range characteristic of RNA tumour viruses. Figure 1 also illustrates the large amount of phosphatase activity present in the cytoplasm of human breast tissue which peaks at a density of 1.11 to 1.13 g cm⁻³ in sucrose gradients, but overlaps into the the 1.16 g cm⁻³ region.

Second, nucleoids or cores that contain the reverse transcriptase and possess a density of 1.26–1.27 g cm⁻³ can be generated from RNA tumour viruses by treatment of virions with non-ionic detergent[8]. By treatment with 1% Sterox-SL of the particulate material with a density of 1.15–1.20 g cm⁻³ from the postmitochondrial supernatant of 50 g of human mammary tumour, we were able to isolate reverse transcriptase activity responding to poly(Cm)·oligo(dG) that banded in the density range of 1.21 to 1.30 g cm⁻³ (peaked at 1.265 g cm⁻³) on a 25–85% (w/v) sucrose gradient (see Fig. 2). This material probably repre-

sents cores of breast particles containing reverse transcriptase. DNA polymerase activity at the top of the gradient represents solubilised enzyme partially dissociated from particulate material and not contained within an intact core[8,9]. Also demonstrated in Fig. 2 is the fact that most of the phosphatase contamination of the particle preparation (seen in Fig. 1) is eliminated by detergent treatment and subsequent equilibrium and velocity centrifugation, so that phosphocellulose chromatography before assay of DNA polymerase activity was not necessary.

The results reported here demonstrate that some human breast tumours, but not non-malignant breast tissues, contain particulate, poly(Cm)·oligo(dG)-directed DNA polymerase activity present in a structure possessing the density of an RNA tumour virus. Some of the DNA polymerase activity can be made to band at a density characteristic of virus nucleoids by treatment with a neutral detergent In the light of these findings and the specificity of poly(Cm)·oligo(dG) for viral reverse transcriptase in other systems[1,4,5], it seems reasonable to conclude that we are detecting the enzyme in human breast tumours. It is clear from our experiment that the problem of phosphatase contamination is serious and must be overcome for successful demonstration of the enzyme. The synthetic probe provides an alternative to simultaneous detection[3] for the identification of RNA-directed DNA polymerase activity in human tissues, and investigations are under way using poly(Cm)·oligo(dG) to search for the enzyme in other human tumours and differentiating tissues.

The significance of particle bound RNA-directed DNA polymerase in human breast cancers is not known. It is conceivable that these particles represent infectious, exogenous aetiological agents of the disease. An alternative hypothesis is that the cancer cell represents a partially differentiated cell that is blocked in further development and continues to express information specific for its developmental state, which could include RNA-directed DNA polymerase associated with specific RNA molecules[1]. The retention of this information, normal for a specific state of development, would then be associated with the specific cancer cell type, and its expression could even be responsible for the malignant state of the cell.

GARY F. GERARD
PAUL M. LOEWENSTEIN
MAURICE GREEN

Institute for Molecular Virology,
St Louis University School of Medicine,
St Louis, Missouri 63110

FRITZ ROTTMAN

Department of Biochemistry,
Michigan State University,
East Lansing, Michigan 48823

1 Green, M., and Gerard, G F., Progress in Nucleic Acid Research and Molecular Biology, 14, 187–334 (1974).
2 Axel, R., Gulati, S. C., and Spiegelman, S., Proc. natn Acad Sci. U.S.A., 69, 3133–3137 (1972).
3 Gulati, S C., Axel, R., and Spiegelman, S., Proc. natn. Acad. Sci. U.S.A., 69, 2020–2024 (1972).
4 Gerard, G F., Rottman, F., and Green, M., Biochemistry, 13, 1632–1641 (1974).
5 Gerard, G. F., Biochem. biophys. Res. Commun , 63, 706–711 (1975).
6 Spadari, S., and Weissbach, A., J. biol. Chem., 249, 5809–5815 (1974).
7 McCaffrey, R., Smoler, D. F., and Baltimore, D., Proc. natn. Acad. Sci. U.S.A., 70, 521–525 (1973).
8 Gerwin, B. J., Todaro, G. F., Zeve, V., Scolnick, E. M., and Aaronson, S A., Nature, 228, 435–438 (1970).
9 Stromberg, K., J. Virol., 9, 684–697 (1972).

Polyadenylic acid sequences in *E. coli* messenger RNA

It is commonly assumed that the messenger RNA (mRNA) of prokaryotes lacks the poly(A) sequences which characterise the 3′ terminus of most mRNA of eukaryotes. A report of the absence of poly(A) sequences from both pulse labelled and stable RNA of *Escherichia coli*[1] is often cited as evidence for such a distinction. This apparent difference has generated speculations ranging from the role of poly(A) sequences in regulating the longevity of eukaryotic mRNA molecules[1], to the evolutionary origin of mitochondria[2] in which poly(A) sequences have been found in a messenger-like RNA[2-5]. Such speculations prompted us to re-examine evidence for AMP-rich sequences in *E. coli* reported[6] before poly(A) sequences were known to be attached to mRNA in animal cells[7-9]. We have found that RNA molecules can be isolated from *E. coli* with the techniques developed for the isolation of poly(A) containing RNA of eukaryotes[10] which have properties of mRNA and contain poly(A) sequence at their 3′ terminus.

Table 1 presents evidence for the presence of poly(A)-containing RNA in *E. coli*. A fraction of total RNA labelled either with a pulse of ³H-adenosine or for 10 min with ³²PO₄ could be bound to oligo(dT) cellulose. Seventy to eighty-five per cent of this RNA could be rebound to oligo(dT) cellulose, showing that nonspecific binding of RNA accounted for little of the RNA bound initially. These data compare favourably with those obtained from HeLa cell mRNA using the same conditions (Table 1, experiment 5). The poly(A) sequences released from these RNA fractions by treatment with ribonucleases and DNase contained about 15% of the total incorporated label showing that the fraction binding to oligo(dT) cellulose (Table 1) was neither free poly(A) nor merely an AMP-rich fragment

In the ³H-adenosine pulse labelling experiments, the fraction of the total RNA binding to oligo(dT) cellulose was considerably lower than would be expected if all mRNA species contained a poly(A) sequence of at least 20 nucleotides which is close to the lower limit of poly(A) length that can be isolated in our conditions (unpublished). In view of the rapid turnover of *E. coli* mRNA and the relatively small size of the poly(A) sequence, variations in the fraction of RNA binding to oligo(dT) may be expected. The average size of poly(A)-containing RNA, as well as the size of the poly(A) sequence itself, tended to vary in different experiments (data not shown). The discrepancy in the fraction of poly(A)-containing RNA binding to oligo(dT) cellulose in the two ³²P-labelling experiments could be explained by the fact that the average lengths of the RNA and its poly(A) sequence in experiment 6 were considerably shorter than in experiment 7 (data not shown).

A rapid turnover of either the poly(A)-containing RNA or the poly(A) sequence is suggested because the poly(A)-containing RNA binding to oligo(dT) cellulose comprised much less of the total stable RNA species of *E. coli* (experiment 4, Table 1).

Figure 1 shows the electrophoretic mobilities of both bound and unbound RNAs in denaturing polyacrylamide gels (98% formamide), as well as the poly(A) sequence released by RNases from the bound RNAs. As Fig. 1*b* shows, bound RNA migrated more slowly than tRNA and almost no label migrated with a mobility similar to the poly-(A) derived from this same RNA fraction. Clearly the failure to find label in gel fractions migrating in the region of the poly(A) sequences means that poly(A) must be covalently attached, since this RNA had been denatured by heating at 60 °C in a large excess of unlabelled poly(A) before its application to denaturing gels. Any measurable labelled poly(A) hybridised to RNA should have been lost through competition with the excess of unlabelled poly(A) present during denaturation.

Figure 1*b* shows that poly(A)-containing RNAs are poly-

Table 1 Isolation of poly(A)-containing RNA from *E. coli*

Experiment		Labelling time (min)	First binding to oligo(dT) cellulose (% total RNA)	Second binding to oligo(dT) cellulose (% first bound RNA)	Poly(A) content (% second bound RNA)
a	³H-adenosine				
	1	0.5	0.23	87.9	17.7
	2	1.0	0.25	81.4	17.7
	3	1.5	0.14	76.1	12.6
	4*	120.0	0.007	68.1	—
	5†	90.0	15.8	93.6	22.4
b	³²PO₄				
	6	10.0	0.16	71.2	17.7
	7	10.0	1.41	86.0	11 5

Table 2 Nucleotide composition of poly(A) sequence

Treatment	Total poly(A) (c.p.m.)	CMP (%)	AMP (%)	GMP (%)	UPM (%)	Recovery (% total poly(A))
RNase T₁	71,000	6.7	82.3	0.7	10.2	100
RNase T₁+RNase A	46,000	2.1	95.6	1.6	0.7	92

Poly(A) was isolated from a poly(A)-containing RNA labelled for 10 min with $^{32}PO_4$. Nuclease treatment as described in Table 1 except that in one case RNase A was omitted. After gel electrophoresis, fractions were pooled as shown in Fig. 2, and the poly(A) recovered was hydrolysed with 0.3 N KOH for 18 h at 37 °C. Nucleotide composition was determined by the method of Osawa *et al.*[19].

disperse, migrating from 4S to 20S. When the logarithm of molecular weight of HeLa 28S, 18S and tRNA which were coelectrophoresed with this RNA and the poly(A), were plotted against their relative mobilities[18], molecular weights of 130,000 and 13,000 were calculated for peak positions of poly(A)-containing RNA and poly(A) respectively, which agreed well with the 11.5% poly(A) content of this RNA.

The polydispersity and greater concentration of these poly(A)-containing RNAs in pulse-labelled RNA suggest

Fig. 1 Electrophoresis of poly(A)-containing RNA and poly(A) in polyacrylamide gels in formamide. Aliquots of RNA and poly(A) from experiment 7 in Table 1 were mixed with 20 μg of non-radioactive poly(A) and ³H-labelled HeLa cell RNA in 98% formamide containing 20 mM NaCl and 10 mM Tris HCl, *p*H 7.6, and heated for 1 min at 60 °C. After cooling at 0 °C, bromophenol blue and sucrose were added. Electrophoresis in 19 cm of 4.2% polyacrylamide gels in formamide was carried out for 5 h at 11 V cm⁻¹ as described[18]. Fractions collected with a Maizel fractionator were counted in a toluene scintillation fluid containing Triton X-100. *a*, Unbound RNA (37,000 cm); *b*, bound RNA (46,000 c.p.m., ○) and poly(A) (5,300 c.p.m., ●) electrophoresed on parallel gels are plotted on the same figure. |—| dye marker; ↓ HeLa 28S RNA (molecular weight 1.9×10^6), 18S RNA (0.71×10^6) and 4S RNA (27,000).

they are species of mRNA although evidence for their translation has not been sought. A relatively small fraction of the total label in the unbound RNA was in ribosomal RNA in these conditions (Fig. 1*a*), showing that labelled mRNA comprised a significant amount of total RNA If at least
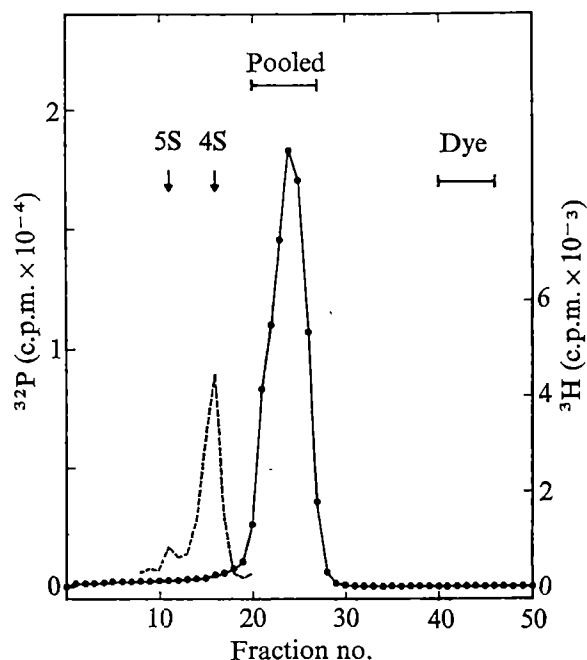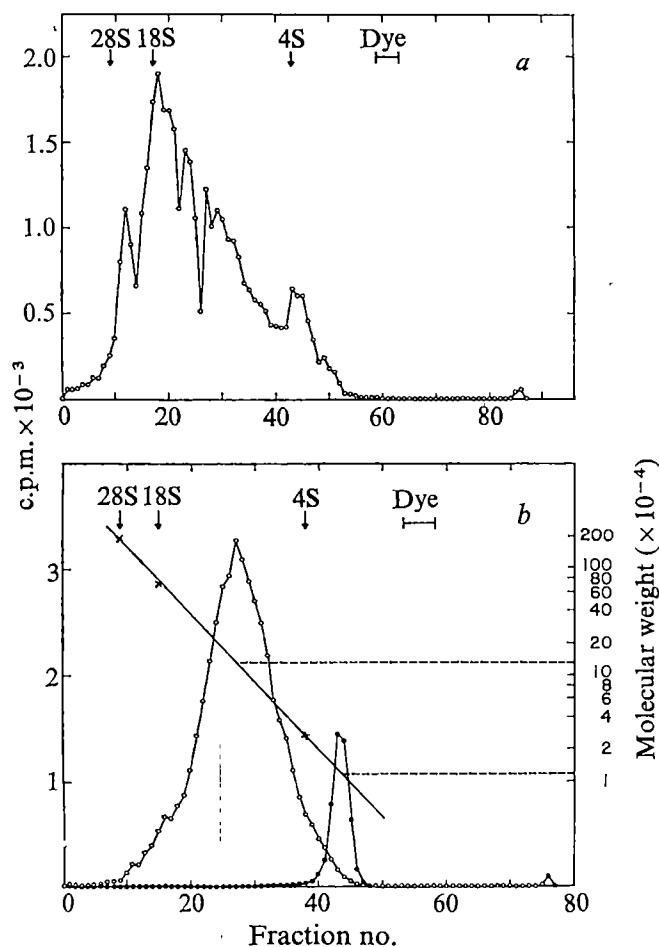
Fig. 2 Electrophoresis of the poly(A) sequence in 10% polyacrylamide gel in formamide. An aliquot of the poly(A) of experiment 7 (Table 1) was coelectrophoresed with HeLa ³H-labelled RNA in a 12 cm, 10% polyacrylamide gel in formamide (98%) at 14 V cm⁻¹ for 4.5 h. After fractionating as in Fig. 1, ^{32}P was measured by Cerenkov radiation. Aliquots of fractions 8–20 were counted as in Fig. 1 to locate ³H-labelled 5S and 4S RNA.

50% of total radioactivity was in mRNA, then poly(A)-containing RNA comprised less than 3% of mRNA.

Figure 2 shows an electrophoretogram of ^{32}P-poly(A) isolated after RNase T₁ treatment of the RNA. Poly (A) again migrated faster than the 4S and 5S RNA of HeLa cells. From its electrophoretic mobility in formamide gel, this ^{32}P-labelled poly(A) sequence was estimated to contain about 45 nucleotides.

The GMP content (Table 2) of the poly(A) sequence from RNase T₁ digested RNA shown in Fig. 2 indicates that poly(A) is near the 3' end of RNA, since there is far less than one GMP residue for a sequence of about 40 nucleotides. When hydrolysed with RNase T₁ and RNase A, the RNA yielded a poly(A) lacking UMP residues. The CMP and GMP residues found in this poly(A) could either be contaminants or nucleotides at the 3' end of the sequence. The first possibility is likely for CMP since the alkaline hydrolysis of poly(A) always produces about 1% of a component which comigrates with CMP during electrophoresis[21].

Absence of these nucleotides from the 3′ end of poly(A) was, however, shown more directly when a RNase T_1 and RNase A digest of RNA from cells labelled with ^{3}H-adenosine released 6,000 c.p.m. in the poly(A) sequence of which 5,200 were recovered in AMP and 210 in adenosine. The average chain length of 25 calculated from these values was compatible with, although not identical to, the length of the ^{32}P-labelled poly(A) estimated by gel electrophoresis in Fig. 2. These data along with those of Table 2 show the poly(A) sequence of *E. coli* to be at the 3′ end of the RNA as is the case for the mRNA of eukaryotic cells[17,20,21].

The detection in *E. coli* of poly(A) sequences covalently bound at the 3′ ends of RNAs with characteristics of mRNA should eliminate speculation on roles for poly(A) sequences which define a function of eukaryotes not shared by prokaryotes, such as transport of mRNA from the nucleus. For the same reason, the presence of poly(A) sequences in mitochondria can no longer be used as evidence against the prokaryotic origin of mitochondria.

The concentration of poly(A) sequences in both pulse and fully labelled *E coli* shows that, in contrast to animal cells and yeast, probably no more than 3% of mRNA molecules contain a poly(A) sequence long enough to bind to oligo(dT) cellulose. This difference is perhaps not surprising since the coupling of translation with transcription in prokaryotes may not allow the accumulation of many mRNAs with completed 3′ termini. The low levels could also account for the failure of Perry *et al.*[1] to detect poly(A) sequences in *E. coli* mRNA, since they are close to the noise level reported for their experiments In addition, the Millipore filter techniques they used would not have bound poly(A) sequences as short as those found here[22,23].

Although only a few mRNA species of *E. coli* have poly-(A) at the 3′ terminus, many mRNAs may have oligo(A) sequences at the 3′ terminus which are too short to bind such RNA to oligo(dT) cellulose. Such adenylated 3′-termini have been found on the mRNAs synthesised *in vivo* after T_7 (ref. 24), as well as Φ80 phage[25] infection of *E. coli*. In both cases a variability in the yield of 3′ adenylated mature mRNA species and the length of the oligo(A) sequence (usually 2 to 5 AMP residues) suggests that adenylation is posttranscriptional. Kramer *et al.*[24] suggested that oligo adenylation of the T_7 early mRNAs is a primitive counterpart of the poly adenylation found in eukaryotic mRNAs.

In considering a function for the polyadenylation of RNA molecules, it is interesting that the precursor of 5S RNA of *E. coli* may contain as many as seven uninterrupted AMP residues at the 3′ end[26]. Because 5S RNA has no known messenger function, a role for adenylation in RNA processing or maturation should perhaps be considered further, particularly since in spite of some recent elegant experiments, a role for poly(A) sequences in mRNA translation in eukaryotes has not been demonstrated[27].

HIROSHI NAKAZATO
S. VENKATESAN
MARY EDMONDS

Department of Biochemistry,
Faculty of Arts and Sciences,
University of Pittsburgh,
Pittsburgh, Pennsylvania 15261

1 Perry, R. P., Kelley, D. E , and LaTorre, J., *Biochem. biophys. Res Commun.*, 48, 1593–1600 (1972).
2 Perlman, S., Abelson, H T., and Penman, S., *Proc. natn. Acad. Sci. U.S A.*, 70, 350–353 (1973).
3 Avadhani, N G., Kuan, M., VanDer Lign, P., and Rutman, R. J., *Biochem. biophys. Res. Commun*, 51, 1090–1096 (1973).
4 Ojala, D., and Attardi, G , *J. molec. Biol.*, 82, 151–174 (1974).
5 Hirsch, M., Spradling, A., and Penman, S., *Cell*, 1, 31–35 (1974).
6 Edmonds, M., and Kopp, D. W., *Biochem. biophys. Res. Commun*, 41, 1531–1537 (1970).
7 Edmonds, M., Vaughan, M. H., and Nakazato, H , *Proc. natn Acad. Sci. U.S.A.*, 68, 1336–1340 (1971).
8 Lee, S Y., Mendecki, J., and Brawerman, G., *Proc. natn. Acad Sci. U S.A*, 68, 1331–1335 (1971)
9 Darnell, J. E., Wall, R., and Tushinski, R. J., *Proc. natn Acad. Sci U.S.A*, 68, 1321–1325 (1971).
10 Nakazato, H., and Edmonds, M., *J. biol Chem*, 247, 3365–3367 (1972), in *Methods in Enzymology*, XXIX, (edit by Grossman, L , and Moldave, K), 431–443 (Academic, New York, 1974.)
11 Gesteland, R F , *J. molec. Biol.*, 16, 67–84 (1966).
12 Nakada, D., *J. molec. Biol* , 12, 695–725 (1965)
13 Imamoto, F., Morikawa, N , Sato, K., Mishima, S., and Nishimura, T., *J molec. Biol.*, 13, 157–168 (1965).
14 Okamoto, K., Sugino, Y., and Nomura, M , *J. molec Biol* , 5, 527–534 (1962).
15 Girard, M., in *Methods in Enzymology*, XII, (edit. by Grossman, L., and Moldave, K), 581–588 (Academic, New York, 1967)
16 Nakazato, H., Edmonds, M., and Kopp, D. W., *Proc natn Acad Sci U.S.A* , 71, 200–204 (1974).
17 Nakazato, H., Kopp, D. W , and Edmonds, M., *J biol. Chem* , 248, 1472–1476 (1973).
18 Staynov, D. Z., Pinder, J. C., and Gratzer, W B., *Nature new Biol.*, 235, 108–110 (1972).
19 Osawa, S., Takata, K., and Hotta, Y , *Biochim. biophys, Acta*, 28, 271–277 (1958).
20 Mendecki, J., Lee, S. Y., and Brawerman, G., *Biochemistry*, 11, 792–798 (1972)
21 Molloy, G. R., and Darnell, J. E., *Biochemistry*, 12, 2324–2330 (1973).
22 Eaton B T., and Faulkner, P., *Virology*, 50, 865–873 (1972).
23 Gorski, J., Morrison, M. R , Merkel, C. G , and Lingrel, J. B , *J. molec. Biol* , 86, 363–371 (1974).
24 Kramer, R. A., Rosenberg, M., and Steitz, J. A., *J. molec. Biol.*, 89, 767–776 (1974).
25 Pieczenik, G., Barrell, B. G., and Gefter, M. L., *Archs biochem Biophys.*, 152, 152–165 (1972).
26 Griffin, B E., and Baillie, D. L., *FEBS Lett.*, 34, 273–279 (1973).
27 *Nature*, 253 308 (1975).

Carbonium ion as ultimate carcinogen of polycyclic aromatic hydrocarbons

RECENT results have indicated that the proximate metabolites of benzo(a)pyrene[1] and benz(a)anthracene[2,3], two typical carcinogenic aromatic hydrocarbons, contain the 1,2-epoxy-3, 4-dihydroxy-tetrahydronaphthalene moiety. This moiety arises from its parent hydrocarbon by formation of an arene oxide, enzymatic ring opening of this oxide to a diol, and then a second epoxidation. As the initially formed arene oxide is opened in a *trans* fashion[4], only two geometrical isomers for the proximate metabolite are possible, and these are depicted as partial structures (I) and (II).
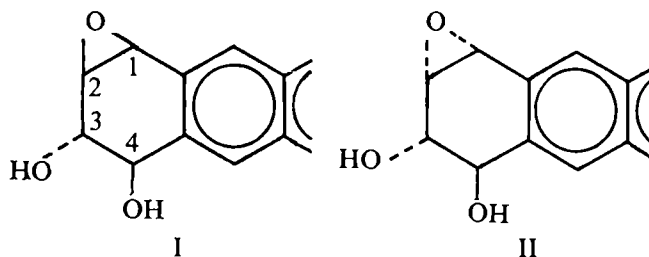
Here I discuss stereochemical features of these two isomers, and indicate that (I) can be predicted to have a much greater chemical reactivity with nucleophiles than (II). This is of interest because of the accepted theory[5,6] that chemical carcinogens act by being metabolised to electrophiles which then react to form covalent linkages with nucleophilic groups present on DNA. Thus it becomes necessary to find metabolites that possess special features which lead to high reactivity in physiological conditions. I describe here that isomer (I) possesses a strategically placed OH group which is expected to assist the epoxide ring opening by neighbouring group participation and thus lead to substantially enhanced reactivity with nucleophiles. Furthermore, this OH group is expected to favour internal ion-pair formation and thus provide an electrophile of the appropriate character to react with DNA *in vivo*.

Both isomers (I) and (II) can exist in two conformations. In each case, however, only one conformation is favoured. The less favoured conformations involve eclipsing of bonds emanating from C_2 and C_3, the more favoured conformation of each isomer is shown in Fig. 1. This shows that the two hydroxyl groups are quasi-axially disposed in (I) and quasi-equatorial in (II). In particular the 4-OH group in isomer (I) is ideally situated for internal hydrogen bonding with the epoxide. This hydrogen bonding leads to a weakening of the C–O bonds on the epoxide and greatly facilitates nucleophilic attack at C_1 or C_2. The interatomic distance from the O of the
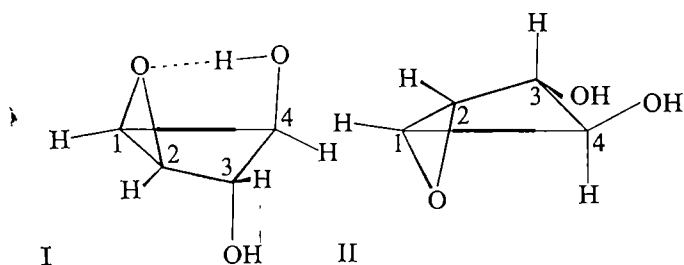
Fig. 1 Preferred conformations of partial structures (I) and (II), isomers of the 1,2-epoxy-3,4-dihydroxy-1,2,3,4-tetrahydronaphthalene moiety of the proximate metabolites of carcinogenic aromatic hydrocarbons. Conformations are viewed from a point equidistant from C_2 to C_3 and towards the molecule Viewing point is in the plane of the aromatic ring but remote from it.

epoxide to that in the 4-OH group is 2.5 Å (Dreiding models). Generally epoxides require external acid catalysis for ring opening; however, with isomer (I) the catalysis will be intramolecular and thus the ring opening will occur rapidly under neutral conditions. In isomer (II), on the other hand, the OH groups are not appropriately oriented and such internally assisted epoxide ring opening is not possible, with the result that this isomer will be much less reactive.

Assistance by OH groups in the opening of epoxides by nucleophiles has been recognised in steroids[7] and in the anti-leukaemic triptolide molecules[8]. In each of these examples, the OH group and the epoxide have the same structural and configurational relationships. These same relationships are also found between the 4-OH group and the 1,2-epoxide in (I). There is also conformational similarity as, in all three molecules, the key OH groups are in axial conformations. O–O interatomic distances of 2.7 Å (Dreiding models) and 3.1 Å (X-ray crystallographic work[9]), respectively, in the steroid and triptolide examples above, indicate that the distance of 2.5 Å in isomer (I) is short enough for strong interaction to take place.

The nucleophilic opening of the epoxide of isomer (I) is depicted formally in (III). Nucleophilic attack is possible at both C_1 and C_2 but attack at C_1 is shown because of the steric hindrance that the axial 3-OH group may cause, and because of the probable formation of the well stabilised internal ion pair (IV), in which the O anion is stabilised by the 4-OH group with the positive carbon centre stabilised by the aromatic nucleus. The positive carbon centre is in effect an aralkyl carbonium ion.

III IV
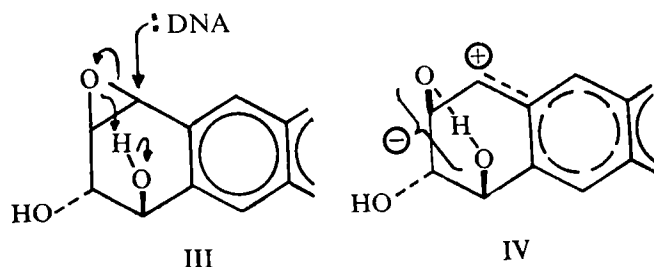
Internal ion pairs such as (IV) have already been shown to occur as the rate-limiting step in the spontaneous rearrangement of arene oxides[10,11] in neutral conditions (which may be accompanied by the NIH shift[12]). In the case of (IV), however, the additional stability provided by participation of the neighbouring 4-OH group is a novel feature. Such additional stability is of course not possible in the analogous zwitterion from (II). The important consequence of the formation of the well-stabilised internal ion pair (IV) from isomer (I) is that this isomer will react with appreciable S_N1 character, that is, as a carbonium ion, rather than by an S_N2 mechanism. As a result it will react with a low sensitivity to the nucleophilic

strength of the incoming nucleophile and small but significant quantities of products from reaction with relatively poor nucleophiles, in the presence of stronger nucleophiles, will result. As DNA has low nucleophilic properties[13], it therefore becomes a necessary feature of an ultimate metabolite that it can indeed react with appreciable carbonium-ion character so that it can alkylate DNA in vivo. The ion pair (IV) will react in this manner and thus has the appropriate electrophilic character to be considered as the ultimate metabolite of aromatic hydrocarbons. By contrast, the isomer (II) as a more typical arene oxide will react with much greater sensitivity to nucleophilic strength[14], that is, in a predominantly S_N2 manner, and alkylate DNA to a negligible extent only. These general concepts have been elaborated previously[15-18].

A more quantitative approach is possible if we arbitrarily assign Swain–Scott[19] substrate constant values s to isomers (I) and (II). By analogy with those other carcinogens which act by way of carbonium ions (for example, isopropyl methanesulphonate[16], $s=0.29$; N-ethyl-N-nitrosourea[17], $s=0.26$; and N-methyl-N-nitrosourea[17], $s=0.42$), the s value of isomer (I) may be 0.3, whereas that of isomer (II) may be 0.9 (for example, glycidol[19], $s=1.00$). Using the Swain–Scott equation[19]

$$\log(k_{GSH}/k_{DNA}) = s(n_{GSH} - n_{DNA})$$

relative rates of reaction with glutathione (a strong biological nucleophile and representative of other SH-containing compounds) and DNA can be compared. Values for the nucleophilicities n of SH groups and DNA are about 5.1 and 2.5, respectively[15], and using these values it may be calculated that isomer (I) would react six times slower with DNA than with glutathione, whereas isomer (II) would react 220 times slower with DNA than with glutathione. Thus isomer (I) will alkylate a small but significant proportion of DNA, whereas isomer (II) will alkylate only a negligible proportion of DNA.

The sites of alkylation on DNA are multiple[1,3], and this also accords with the concept that the ultimate metabolite must have appreciable carbonium-ion character and consequently show poor discrimination for nucleophiles. A general value for the nucleophilicity of DNA was taken above, as it is not possible at this stage to consider any particular sites on DNA as critical. Aromatic hydrocarbons act by causing frame-shift alterations[20,21], in contrast to carbonium ions of smaller molecular size which cause base substitutions. With agents of this latter class, the very weakly nucleophilic[16] O^6 group of guanine may be a critical alkylation site[16,22]. Alkylation by a small group at this site interferes with DNA interstrand hydrogen bonding and base substitution occurs[23]. Alkylation by large groups such as (IV) will clearly interfere more profoundly with the conformation of the double helix, by intercalation (see below) or by a 'base-displacement' mechanism[24], with frame-shift alterations resulting.

The extent of alkylation of DNA in the presence of other cellular constituents such as glutathione will depend not only on the first-order reaction rates described above, but also on the concentration of DNA and glutathione in the immediate vicinity of the ultimate metabolite. As the planar aromatic structure of the rest of the carcinogen molecule is expected to be predominantly intercalated[20,21] with DNA, the concentration of DNA nucleophilic sites will be high compared to SH glutathione and protein sites. The ultimate metabolite thus has characteristics of an affinity label[25] for DNA, in that non-covalent binding encourages more specific covalent binding. The suggestion that many carcinogens and frame-shift mutagens may intercalate and then react covalently has been made[20,21,26-28].

The toxicities of bromobenzene and aromatic hydrocarbons are both mediated by the formation of an arene oxide metabolite, but whereas large amounts of bromobenzene are required to produce hepatotoxicity and this is seen only when glutathione is depleted[29], only very small amounts of aromatic hydrocarbons are required for a carcinogenic effect and no similar

threshold dose exists. This paradox can now be understood in the framework of the above discussion. Thus the 3,4-oxide of bromobenzene reacts in an S_N2 fashion, that is, selectively with strong nucleophiles such as glutathione. Only when body glutathione is depleted by reaction with an equivalent of bromobenzene oxide will this oxide react in appreciable quantity with the next most nucleophilic groups, which are on proteins. The toxicity arises as a result of this covalent reaction with proteins[29]. DNA, a poor nucleophile, is presumably not attacked in the presence of proteins. On the other hand, I have described here how the arene oxide (I) from aromatic hydrocarbons can react as a carbonium ion with DNA, in the presence of glutathione and proteins, and thus no threshold need exist.

PETER B. HULBERT

Department of Pharmaceutical Chemistry,
The University of Bradford,
Bradford, Yorkshire BD7 1DP, UK

1 Sims, P., Grover, P. L., Swiasland, A., Pal, K., and Hewer, A., *Nature*, 252 326–328 (1974).
2 Booth, J., and Sims, P., *FEBS Lett.*, 47, 30–33 (1974).
3 Swaisland, A. J., *et al.*, *FEBS Lett.*, 47, 34–38 (1974).
4 Jerina, D. M., Ziffer, H., and Daly, J. W., *J. Am. chem. Soc.*, 92, 1056–1061 (1970).
5 Miller, J. A., *Cancer Res.*, 30, 559–576 (1970).
6 Miller, E. C, and Miller, J A., *Pharmac. Rev.*, 18, 805–838 (1966).
7 Barton, D. H. R., and Houminer, Y., *J. chem. Soc , Chem. Commun.*, 839–840 (1973).
8 Kupchan, S. M., and Schubert, R. M., *Science*, 185, 791–793 (1974).
9 Gilmore, C J., and Bryan, R. F., *J. chem. Soc. Perkin II*, 816–819 (1973).
10 Kasperek, G. J., Bruice, T. C., Yagi, H., and Jerina, D.M., *J. chem. Soc., chem. Commun.*, 784–785 (1972).
11 Kasperek, G J., Bruice, P Y., Bruice, T. C., Yagi, H. and Jerina, D. M., *J. Am. chem. Soc.*, 95, 6041–6046 (1973).
12 Daly, J. W., Jerina, D. M., and Witkop, B., *Experientia*, 28, 1129–1149 (1972).
13 Walles, S., and Ehrenberg, L., *Acta chem. scand.*, 23, 1080–1082 (1969).
14 Jeffrey, A. M., *et al.*, *J. Am. chem. Soc.*, 96, 6929–6937 (1974).
15 Osterman-Golkar, S., Ehrenberg, L., and Wachtmeister, C. A., *Radiat. Bot.*, 10, 303–327 (1970).
16 Lawley, P. D., Orr, D. J., and Jarman, M., *Biochem. J*, 145, 73–84 (1975).
17 Veleminsky, J., Osterman-Golkar, S., and Ehrenberg, L., *Mutat. Res.*, 10, 169–174 (1970).
18 Osterman-Golkar, S., *Mutat. Res.*, 24, 219–226 (1974).
19 Swain, C. G., and Scott, C. B., *J. Am. chem. Soc.*, 75, 141–147 (1953).
20 Ames, B. N., Sims, P., and Grover, P. L., *Science*, 176, 47–49 (1972).
21 Ames, B. N., Durston, W. E., Yamasaki, E., and Lee, F. D., *Proc. natn. Acad. Sci. U.S.A.*, 70, 2281–2285 (1973).
22 Loveless, A., *Nature*, 223, 206–207 (1969).
23 Gerchman, L. L., and Ludlum, D B., *Biochim. biophys. Acta*, 308, 310–316 (1973).
24 Levine, A. F., Fink, L M., Weinstein, I. B., and Grunberger, D., *Cancer Res.*, 34, 319–327 (1974).
25 Shaw, E., in *The Enzymes*, 1 (edit. by Boyer, P. D.), third ed., 91–146 (Academic, London and New York, 1970).
26 Ames, B. N., Gurney, E. G., Miller, J. A., and Bartsch, H., *Proc. natn Acad. Sci. U.S.A.*, 69, 3128–3132 (1972).
27 Creech, H. J., Preston, R. K., Peck, R. M , O'Connell, A. P., and Ames, B. N., *J. med. chem.*, 15, 739–746 (1972).
28 Hartman, P. E., and Hulbert, P. B., *J. Toxic. Environ Hlth* (in the press).
29 Jollow, D. J., Mitchell, J R., Zampaglione, N., and Gillette, J. R., *Pharmacology*, 11, 151–169 (1974).

Alternatives to the use of tabulated values of distributions in statistical programmes

SPECIFIC values from tables of statistical distributions that vary with degrees of freedom and probability (for example Student's t, χ^2, correlation (r) or variance ratio (F)) are often included as input data, even to large detailed statistical packages[1,2]. Alternatively, the complete tables are held in store. The former makes the program less flexible while the latter uses store inefficiency and is impractical for desk-top computers. A common alternative practice is to use approximate values; thus for example it has been suggested[3] that for a sample number of thirty and more the values of t should be 2.0 when the probability (P) is 0.05. This value for t (30–∞) is within ±2% of the true value and was considered 'a close approximation [suitable] for most purposes'.

The alternative I propose is that simple equations should be formulated to calculate values for such statistical distributions to conserve store space and to make programs more adaptable. The equations should be relatively simple and within the present capabilities of small fixed-function desk-top computers.

As an example, the equation (1) is proposed as a simple approximate solution of Student's distribution of t. It was obtained by a trial and error fitting of the best equation-type

and curve to a graph of the distribution data. In this equation t_{Pn} is the value of t when the probability is P, for example 0.05, and the sample number is n, thus:

$$t_{Pn} = [\exp(k_P/(n-1))] + t_{P\infty} - 1.0 \qquad (1)$$

in which the distribution of $t_{P\infty}$ is given by:

$$t_{P\infty} = -0.1093\,(\log_{10}(1/P))^2 + 1.26\,\log_{10}(1/P) + 0.4941 \qquad (2)$$

The equation giving the values of k_P used to obtain optimum values of t in equation (1) over the range of probability 0.001–0.1 and using the values of $t_{P\infty}$ from equation (2) is given by:

$$k_P = [2.7497\,\log_{10}(1.0/P)] - 1.1994 \qquad (3)$$

This formula (1) provides values of t to within ±5% of the true value[4] for a sample number of between three and infinity and within the range of probabilities $P = 0.1$–0.005; values of t for a sample number of thirty and over are within ±1% of the true t. Values of t over the wider probability range of $P = 0.2$–0.001 were within ±8% of the true t for sample numbers of four to infinity; sample numbers over 30 were within ±3% of the true t.

I realise that when the confidence or fiducial limits thus obtained do not show a clear decision the true tables must be consulted or the equation suggested must be refined; but in most cases these small errors are of little consequence.

The correlation coefficient, r, can also be calculated knowing t for n pairs of samples at the probability P using the equation below (modified from Fisher and Yates[5]):

$$r^2 = t^2/[(n-2)+t^2] \qquad (4)$$

Answers to statistical problems are usually presented in terms of a few standard values of probability, for example the t test confidence intervals. An iterative process could be used with suitable formulae to express the differences between sets of samples in the form of an exact probability, for example $P = 0.06$. With the present formula, results are only reliable within the range of probability 0.1 to 0.005. The use of an approximate formula to calculate the critical values of F could then result in a matrix of probabilities for a comparison of several sets of data. With desk-top computers, the latter step will depend on the demand to make statistical distributions available as fixed functions.

F. H. DAWSON

Freshwater Biological Association,
River Laboratory, East Stoke,
Wareham, Dorset BH20 6BB, UK

1 Sokal, R. R , and Rolf, F. J., *Biometry* (Freeman, San Francisco, 1969)
2 Davies, R. G., *Computer programming in Quantitative Biology* (Academic Press, London, 1971)
3 Elliott, J. M., *Statistical Analysis of Samples of Benthic Invertebrates* (Scientific Publication No. 25., Freshwater Biological Association, Ambleside, 1971)
4 Snedecor, G W, and Cochran, W. G , *Statistical Methods* (Iowa State University Press, Ames, Iowa, 1968)
5 Fisher, R. A , and Yates, F., *Statistical Tables for Biological, Agricultural and Medical Research* (Oliver and Boyd, Edinburgh, 1953).

Erratum

In the article "Binding of flexible ligands to macromolecules" by A. S. V. Burgen, G. C. K. Roberts and J. Feeney (*Nature*, 253, 753; 1975), the equation at the foot of the first column on page 754 should read.

$$\Delta F_1 = -RT\ln K_1 = \Delta H_1 - T(\Delta S_{tr} + \Delta S_{rot} + \Delta S_{conf.\,1})$$

and not as printed.

matters arising

Origin of cellular senescence

I HOPE to clarify a point regarding the recent letter of Dykhuizen[1]. He speculated that cellular senescence is the result of a selection process imposed by an organism's need to ensure its survival by limiting the size of atherosclerotic plaques which, he believed, originated from relocated fibroblasts. This is an interesting new view of the limit on fibroblast doubling potential described by Hayflick[2] and others, and the predictions he derives from the hypothesis can be tested experimentally. These include the ultimate cessation of plaque growth when cells at the edge senesce, higher division potential of cells isolated from the centres of plaques, and a correlation between final plaque size and the age of the organism at the plaque's initiation. The fulfilment of these predictions will shed some light on the (probably fibroblastic) origin of plaques but is not critically dependent on an evolved origin for cellular senescence (or doubling potential limit) in the manner Dykhuizen suggests.

The expected observations could be explained, without resort to an evolutionary origin, simply on the basis of known facts. Specifically, cellular senescence manifests itself through a limit on the doubling capacity of fibroblasts; such cells are subject to "contact inhibition" of division[2], and the doubling potential of fibroblasts is related to the age of the donor[3]. Whatever the ultimate cause of cellular senescence or its origin, the behaviour, given these facts, will be the same if plaques do arise from fibroblasts.

The predictions thus do not differentiate between Dykhuizen's explanation of an evolved origin for cellular senescence and a number of other processes which have been postulated as the primary cause of ageing such as error accumulation, a specific genetic programme or the exhaustion of a developmental programme.

ROY WIDDUS

The Center for Theoretical Biology,
State University of New York/Buffalo,
4248 Ridge Lea,
Amherst, New York 14226

1 Dykhuizen, D., *Nature*, 251, 616–618 (1974)
2 Hayflick, L., *Expl Cell Res.*, 37, 614–636 (1965)
3 Martin, G. M., Sprague, C. A., and Epstein, C. J., *Lab. Invest.*, 23, 86–92 (1970).

DYKHUIZEN REPLIES—The lack of an understandable selective advantage for cellular senescence has been a major criticism of a hypothesis for its evolutionary origin[1]. The main point of my paper[2] was to provide a reasonable evolutionary explanation. To do this, I presented a novel theory of the formation of atherosclerotic plaques. The predictions were meant to test this theory which, if correct, implies that the size of plaques is limited by cell senescence, providing a possible selective advantage to the organism.

Given the assumption that a specific genetic programme could only arise through evolution, the evolutionary origin of cell senescence could be demonstrated by finding a gene, which, when rendered non-functional, allows cells which would normally senesce to become immortal. The error and evolutionary hypotheses could possibly be distinguished by determining the percentage of daughter cells of recently divided cells which go on to divide in a senescent culture and in an immortal culture derived from otherwise similar cells. Orgel[1] suggested that if his error theory holds, then both percentages should be near 50%—slightly less in the senescent and slightly more in the immortal culture. If the hypothesis of evolutionary origin is correct, these percentages should be quite different, say, 20% and 90% respectively.

Australian National University.

1 Orgel, L. E., *Nature*, 243, 441–445 (1973).
2 Dykhuizen, D., *Nature*, 251, 616–618 (1974).

Further note on electron diffraction symmetries

IN connection with diffraction symmetries it has been suggested[1] that there are two conditions for a centrosymmetric pattern though there is some contention that the first of these conditions is incomplete. As the chief protagonists in this debate we wish to clarify the situation with respect to zero-beam symmetry and reciprocity.

We shall consider first those symmetries observable in convergent-beam zone-axis patterns, that is, restricted sections of reciprocal space distributions disposed on a plane with the geometry of the diffraction pattern. Under these restrictions, a rotation diad along the zone axis completely determines a centrosymmetric pattern for all beams, that is, it is a sufficient condition for the whole pattern and does not require any consideration of reciprocity. (Note that to preserve the diad symmetry consideration must be restricted to either parallel-sided foils lying perpendicular to the incident beam, or else to crystals with boundaries which conform to the diad symmetry.)

This covers the example given by Steeds[2] and requires a correction to the table presented by Goodman[7]. To the last column of entry (2) in the table should be added the words "unless there is a rotation diad parallel to the zone, in which case the pattern will be centrosymmetric". Consideration of reciprocity allows general conditions to be found for zero-beam symmetry which do not apply to the remainder of the pattern as a whole but also give the internal symmetry of each dark-field intensity distribution. Thus, a central mirror or glide plane (condition 1 of Goodman[1]) is a sufficient condition for centrosymmetry in the zero-beam pattern, but not in the remainder of the pattern. For other symmetries, a rotation diad or twofold screw axis, midway between the surfaces and parallel to them, ensures a mirror line in the zero-beam pattern (though not in the remainder of the pattern), perpendicular to the diad or screw axis (though this does not ensure a centre of symmetry in the zero-beam pattern). Higher symmetries than a mirror line or a centre can be built up by combination. Derivation of these rules from the invariance properties of the crystal does not require an explicit solution for the wave function, and can be applied to a crystal of any structure or surface geometry.

From such rules one can proceed to derive a description of a zone-axis pattern for a particular space group. But considerations of electron diffraction always concern crystals that are finite in at least one direction, and the strong interaction of electrons with the crystal potential makes it impossible to ignore the boundary conditions, so the symmetry of the total crystal must, therefore, be considered. The influence of tilted surfaces may be avoided to any accuracy by choosing crystal foils sufficiently parallel-sided and horizontal (say in the range of 0–20°). But the influence of crystal termination in a flat horizontal specimen will mean that the crystal may not have the three-dimensional symmetry of the space group, and in such cases the crystal boundaries used in a symmetry derivation need to be stated.

Experimental limitations need to be considered. Convergent-beam diffraction, and diffraction-contoured micrographs, are practical methods of symmetry analysis with individual limitations. The pattern symmetries can be expressed by two-dimensional point-group symbols, whether investigating two-dimensional projection symmetries or three-dimen-

sional symmetries of the crystal. In the first approximation, the two-dimensional symmetry of the projection at the zone determines the diffraction symmetry and the symmetry of the pattern only has the symmetry of the projection. When measurements are sufficiently accurate, or cover a sufficient angular range, the symmetries must be derived from the three-dimensional space group in the way indicated.

P. Goodman

CSIRO, Division of Chemical Physics,
PO Box 160, Clayton,
Victoria, Australia 3168

J. W. Steeds

H. H. Wills Physics Laboratory,
Royal Fort, Tyndall Avenue,
Bristol, BS8 1TL, UK

1 Goodman, P., *Nature*, 251, 700–701 (1974).
2 Steeds, J. W., *Nature*, 251, 701–702 (1974).

Binding of 25-hydroxy vitamin D_2 to plasma protein in New World monkeys

Belsey *et al.*[1] have shown that the lower potency of ergocalciferol (D_2) compared with cholecalciferol (D_3) in chicks is reflected in a weaker binding of D_2 metabolites to the plasma transport proteins. Further investigations in other species known to be resistant to D_2 were recommended[1] to establish whether similar correlations exist between biological activity and binding to transport proteins. Since New World primates (Cebidae) utilise D_2 less efficiently than D_3 (refs 2–4) we examined the transport proteins in four species of New World monkeys to test whether they also discriminate against D_2 metabolites.

A competitive protein binding assay involving the displacement of ^{3}H-25-OH-D_3 from the specific plasma transport proteins by 25-OH-D_2 and 25-OH-D_3 was used to test the relative binding of these two metabolites of D_2 and D_3.

In the assay system with plasma from the monkey *Cebus albifrons*, increasing amounts of 25-OH-D_2 and 25-OH-D_3 produced similar reductions of the bound ^{3}H-25-OH-D_3 (Fig. 1) indicating equivalent binding of the two steroids by the *C. albifrons* transport protein. A similar result was obtained for the three other primates tested—*C. capucinus, Aotus trivirgatus,* and *Callithrix jaccus.* Old World monkeys (Cercopithecidae) utilise D_3 and D_2 with equal efficiency[3] and in the three Old World primate species we studied (*Erythrocebus patas, Macaca mulatta,* and *Papio anubis*) the transport proteins exhibited equal affinity for 25-OH-D_2 and 25-OH-D_3.

There are differences in the type of protein used by the species examined in this study for vitamin D transport. *C. albifrons* and *C. capucinus* use albumin[6] whereas the two other New World and
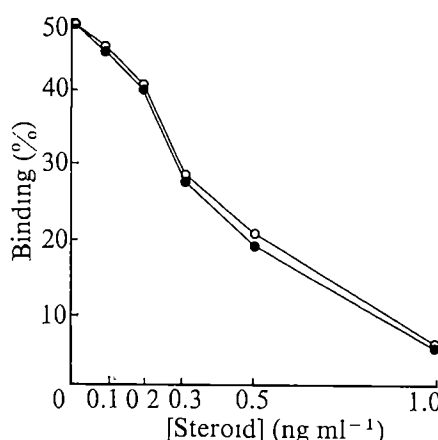
Fig. 1 Competitive displacement of ^{3}H-25-OH vitamin D_3 from *C. albifrons* plasma with increasing amounts of non-labelled vitamin D analogues. Each point is the mean of two determinations ○, 25-OH-D_2; ●, 25-OH-D_3.

the three Old World primates use an α-globulin fraction[6]. The seven primates studied only have a single transport protein[6] whereas the chick has two proteins which exhibit β-globulin mobility on gel electrophoresis[6,7]. Although two types of protein are used for vitamin D transport in these primates their binding properties for 25-OH-D_2 and 25-OH-D_3 are similar.

The results obtained for the steroid binding properties of the New World monkey transport proteins are in contrast to those of the chick proteins which do discriminate against D_2 metabolites[1], an observation we were able to confirm. From this, it would seem that the resistance of New World primates to vitamin D_2 is a result of other differences. These may involve inefficient binding of D_2 and 25-OH-D_2 by intracellular vitamin D binding proteins in liver or kidney respectively or discrimination against the active kidney metabolite 1,25-$(OH)_2$-D_2 by the binding proteins present in target organs such as muscle, gut and bone.

The variations between the binding properties of New World primate and chick transport proteins for 25-OH-D_2 illustrate the difficulty of postulating a general theory of D_2 resistance. These differences confirm that the process of discrimination against D_2 in species resistant to this vitamin cannot be explained uniformly by the properties of the plasma protein as evidenced by the chick. It seems, therefore, that a wider survey of vertebrates known to be resistant to D_2 is required to correlate the random observations made in individual species.

A. W. M. Hay
G. Watson

Nuffield Institute of Comparative Medicine,
The Zoological Society of London,
Regent's Park,
London NW1, UK

1 Belsey, R. E., DeLuca, H F , and Potts, J. T Jr, *Nature*, 247, 208–209 (1974).
2 Hunt, R D., Garcia, F G., Hegsted, D M , and Kaplinsky, N , *Science*, 15, 943–945 (1967)
3 Hunt, R D., Garcia, F. G , and Hegsted, D M , *Lab Animal Care*, 17, 222–234 (1967)
4 Lehner, N. D. M , Bullock, B. C., Clarkson, T B , and Lofland, H B., *Lab. Animal Care*, 17, 483–493 (1967)
5 Edelstein, S., Charman, M , Lawson, D. E M , and Kodicek, E., *Clin Sci molec. Med.*, 46, 231–240 (1974)
6 Hay, A. W. M , and Watson, G , *Comp. Biochem. Physiol.* (in the press)
7 Edelstein, S , Lawson, D E. M , and Kodicek, E , *Biochim. biophys. Acta.*, 270, 570–574 (1972).

Ultraviolet viewing with battery-operated television camera

Lavigne and Oritsland[1] have shown that ultraviolet photography can be used usefully for the detection of white animals on snow. Polar bears, for example, ordinarily match their background and may be difficult to spot, certainly from a distance, but when photographed in the near ultraviolet band of the electromagnetic spectrum (300–400 nm) they appear as black bodies, sharply contrasted against the snow. The technique is said to have application for the remote sensing of polar bears in the field. We suggest that ultraviolet detection can also be carried out conveniently with a television camera. Such cameras are intrinsically sensitive to the near ultraviolet band, and to be used for ultraviolet viewing need only be outfitted with an ultraviolet-transmitting lens and filter. We have used the technique for viewing ultraviolet contrast patterns on flowers and butterflies[2], but it should also lend itself to detection on snow of animals which absorb in the ultraviolet. Though conventional, portable video systems, suitable for use in the field, include both a camera and a recorder, the recorder can be disconnected easily, and the camera can be converted to a self-contained viewer if it is fitted with a battery pack attached to the housing. The disconnection of the recorder decreases the vertical resolution of the camera's monitor (because of the loss of scan synchronisation pulses from the recorder), but the quality of the image is adequate for most purposes (full resolution can be restored by the installation of an oscillator and appropriate divider circuits on the camera). Such cameras provide compact, manageably light, and relatively inexpensive, ultraviolet image transducers.

Daniel J. Aneshansley
Thomas Eisner

School of Electrical Engineering and
Section of Neurobiology and Behaviour,
Cornell University,
Ithaca, New York 14853

1 Lavigne, D. M , and Oritsland, N. A., *Nature*, 251, 218 (1974).
2 Eisner, T., Silberglied, R., Aneshansley, D., Carrell, J E., and Howland, H. C , *Science*, 166, 1172 (1969).

reviews

THERE comes a stage in the development of any powerful new experimental technique, microscopical or otherwise, when its value for research is so well established that an authoritative survey is essential to its full exploitation in science and technology generally, beyond the circle of enthusiastic pioneers. Transmission electron microscopy has long reached that position, and the need is being satisfied by a number of monographs and treatises, including Professor Hayat's *Basic Electron Microscopy Techniques* (a conveniently short volume) and his *Principles and Techniques of Electron Microscopy: Biological Applications*, published by the same house. Whether his *Electron Microscopy of Enzymes* (two volumes, so far) is equally timely I am not qualified to judge. But I am in little doubt that a similar treatment of the biological applications of scanning electron microscopy is at best premature and at worst likely to be misleading. There simply has not been enough work done in the subject to serve as basis for it. Only a limited number of biologists throughout the world are seriously applying themselves to the problems of specimen preparation and observation, as distinct from the exploratory use of the instrument.

However useful it may be to newcomers in the field, the title borne by Hayat's new series covering scanning electron microscopy is a misnomer. In effect, it is a journal of biological scanning electron microscopy, largely devoted to articles describing the work of the individual contributors. True, there is an article descriptive of the instrument itself, compressed into 42 pages and failing to mention the two major treatments by Hearle *et al.* (1972) and Oatley (1972). There are also excellent descriptions of the critical point drying technique (60 pp.) and of the preparation of stereomicrographs (14 pp.), both of which are deserving of a wider readership. Nearly all the remaining 19 articles are short accounts (averaging 17 pp.) of special applications of the instrument in the study of spores, leaf surfaces, plant cell walls, wood, intracellular structures, marine teleosts, ciliated epithelia, lung, bone, and so on. To do him justice, the editor is quite frank in his preface to the second volume: "This treatise [*sic*] departs from the tradition that a book on methodology presents only the contemporary consensus of knowledge. It is written by scholars [who have] anticipated the potential usefulness of a new method."

Microscopy
V. E. Cosslett

Principles and Techniques of Scanning Electron Microscopy: Biological Applications. Edited by M. A. Hayat. Vol. 1: Pp. 273; £11.95. Vol. 2: Pp. xii+171; £10.60. (Van Nostrand Reinhold: New York and London, October 1974.)

Principles and Techniques of Electron Microscopy: Biological Applications. Edited by M. A. Hayat. Vol. 3: Pp. xii+321; £11.35. Vol. 4: Pp. xi+216; £10.60. (Van Nostrand Reinhold: New York and London, November 1974.)

If I had to advise a library on its buying policy, my opinion would be that Volume 1 may be worth its price as an introduction to the subject if *The Use of the Scanning Electron Microscope* by Hearle *et al.* is not already on the shelves, but that Volume 2 should only be bought if urgently requested by local biologists. They would do better, however, to consult the original papers in their field of interest, most, if not all, of which they will find listed in the *Bibliography on Biomedical Applications of Scanning Electron Microscopy* compiled by Boyde *et al.* and published in *Scanning Electron Microscopy* 1973, the proceedings of one of the annual meetings on the subject at the Illinois Institute of Technology.

Principles and Techniques of Electron Microsopy: Biological Applications (Volumes 3 and 4) can be viewed more favourably. This series, now planned to extend to eight volumes, sets out to cover the whole range of knowledge involved, including the operation of the microscope itself in its various modes.

The current volumes contain seven and eight articles, respectively. They vary greatly in nature and style, from a broad survey of a topic to a monograph on an author's special field of work. Understandably, the latter form is usually the more readable and useable, and includes articles on high resolution, dark-field microscopy, in-focus phase contrast and stereological techniques in Volume 3, and in Volume 4 on optical shadowing, relative mass determination in dark-field, microscopy of single cultured cells, denaturation mapping of DNA and the examination of polysomes from cardiac muscle. At the other extreme lie collections of recipes for specimen preparation: selective staining of molecules, sub-

cellular fractionation in the ultracentrifuge, and critical point-drying (surely the hypen is misplaced?) in Volume 3; and ultramicroincineration in Volume 4. The latter volume also contains a comprehensive if uncritical account of preparatory [*sic*] methods for electron probe analysis by X-ray spectrometry. Two articles fall outside these categories: a general introduction to the electron microscope and its operation in Volume 3 and an account of methods for counting virus particles in Volume 4.

Granted that the subject is now so wide ranging as not to be encompassed in a treatise by a single author, the best method of covering it by multi-authorship has to be solved. The treatment planned by the editor of this series falls somewhere between two others now becoming available: the encyclopaedic *Methodensammlung der Elektronenmikroskopie* (Wissenschaftliche, Stuttgart) and *Practical Methods in Electron Microscopy* (North-Holland, Amsterdam). The former breaks down electron microscopy into a great number of sub-topics, treated at a high level of expertise in relatively short compass, whereas each volume of the latter comprises two or three long articles in which a rather broader subject is dealt with in greater depth. Dr Hayat's series covers much the same subject matter, but unevenly and rather randomly. Apart from the first two volumes, which described basic preparative techniques, the two under review (and the remaining four as listed) contain an unrelated assortment of contributions. Some of these are by acknowledged leaders in a subject, others by recipe collectors. The level of approach varies from elementary to advanced; the proof reading varies from excellent to execrable (even the editor's name is misspelt in one place); and the current-awareness varies from yesterday to the day before. In such a rapidly moving subject it is regrettable to find few references in Volume 3 later than 1970 or in Volume 4, later than 1971, apart from cross-references within the series.

That said, these volumes have the merit of purveying in compact form a great deal of information, some of value to present practitioners and most of help to the beginner. He, she or they are likely to decide that one or the other of them is sufficiently valuable to be bought, even at these rather high prices. The whole series ought to be available in any biological laboratory making regular use of the electron microscope. □

Welcome addition to multivariate analysis

Discrete Multivariate Analysis: Theory and Practice. By Yvonne M. M. Bishop, Stephen E. Fienberg and Paul W. Holland. Pp. x+557. (MIT Press: Cambridge, Massachusetts, and London, March 1975.) $27.50.

DISCRETE data, in which each variable takes one of a limited number of possible forms, arise frequently in many fields. Multivariate analysis of such data is required when the joint distribution of several variables is to be studied. The present book gives an account of statistical methods for these problems. It concentrates largely, although not entirely, on an approach in which the log frequencies in cells of a multi-dimensional contingency table are represented in some simple form, the so-called log linear model. To an appreciable extent the book is based on the authors' earlier important contributions to this topic.

The discussion is lucid and very leisurely, excellently illustrated with applications drawn from a wide variety of fields. A good part of the book can be understood without very specialised statistical knowledge. It is a most welcome contribution to an interesting and lively subject.

After an introductory chapter, structural models for multidimensional discrete data are described, and then maximum likelihood methods of estimation are developed; this is followed by a consideration of the methods used for testing degree of fit. Methods for incomplete data (missing cells) are discussed at length. There is an interesting chapter on estimating the size of a closed population, in which the usual methods for the analysis of a multiple capture–recapture census are modified to take account of possible dependencies between the samples on successive occasions. Further chapters include discussions on Markov models for assessing change and on the special problems of 'square' tables. The book ends with two chapters on approaches other than that of the log linear model and chapters on pseudo-Bayes estimates (that is, on smoothing tables), on sampling models and on mathematical aspects.

The scope of the book is rather less than might have been expected from its title. For instance, there is no discussion of latent structure analysis or of the difficult problems of analysing multivariate discrete data in connection with numerical taxonomy.

I have one general criticism, which, though to some extent a matter of taste, seems to me important. The authors barely mention the distinction between response variables (dependent variables) and explanatory variables (independent variables). Yet in quite a number of their examples the point at issue seems to concern how variation in one of the variables is explained by the other variables, which is a univariate and not a multivariate problem. Even if the detailed analysis is little affected, incisive explanation of conclusions is much easier for univariate problems. At a more technical level the authors have ignored the fact that in some—but not, of course, all—of their problems conditioning on ancillary statistics is possible. **D. R. Cox**
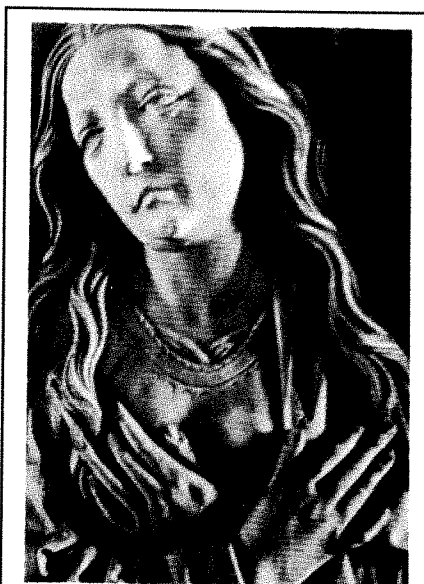
The Virgin Mary, hands crossed in the gesture of humble submission. A wood carving from the Marienalter of Herrgottskirche in Creglingen, Germany. Photograph taken from *Silent Language*, by Macdonald Critchley. Pp. vi+231. (Butterworth: London, April 1975.) £9.50.

Fundamentals

Fundamentals of Mathematics. Vol. 1: *Foundations of Mathematics/The Real Number System and Algebra.* Pp. x+549. Vol. 2: *Geometry.* Pp. xi+685. Vol. 3: *Analysis.* Pp. xiii+541. Edited by H. Behnke, F. Bachmann, K. Fladt and W. Suss. (MIT Press: Cambridge, Massachusetts, and London, 1974.) $15.95 each volume; $40.00 the set.

THIS work stands alone; it is a unique survey of the whole field of pure mathematics, with emphasis on the basic ideas and their interrelationships. For its origin, we must go back over 20 years. In 1954 the International Commission for Mathematical Instruction, at a meeting in Paris, chose the scientific foundations of instruction in mathematics as one topic for the 1958 International Congress in Edinburgh. As a result, the German section of the Commission began Volume I of this work. During its subsequent development authors from various European countries contributed, and it became clear that the material would be important not only for teachers and lecturers but also for research mathematicians. The original publication was in German, and the volumes now reviewed consist of a translation based on the second German edition of 1962; it incorporates various improvements and exercises designed to bring the account up to date.

Each chapter has been written by two authors, one a university research mathematician, the other "a teacher of long experience in the educational system". As a result, over 80 authors have contributed.

Volume I consists of a part on the foundations of mathematics and a part on arithmetic and algebra. Volume II deals with geometry, and consists of 16 chapters covering both the foundations and the analytic treatment of this aspect of mathematics. Worthy of special mention are the chapters on geometrical constructions and on polygons and polyhedra. Volume III is concerned with analysis and includes 14 chapters.

No mathematical specialist can consult the work without becoming acutely aware of the tremendous breadth of mathematical thought and discovery. The student, contemplating research in some area of the subject, would do well to read, in these volumes, of the relationship between that particular area and the subject at large; for it is a distinctive feature of the work that it emphasises the connections between different branches of mathematics.

Many people, including a lot of mathematicians, find it difficult to see perspective in mathematics. To such, the last chapter, on the changing structure of modern mathematics, is to be commended. Here is a very clear account, carefully traced, of what has been happening in recent decades. It is all summed up in the expression "axiomatic way of thinking". There is reference, of course, to the Bourbaki movement in France, and to the development of the axiomatic method in geometry. Then comes the change in algebra, namely the breakthrough of axiomatic algebra (covered particularly well by E. Artin and Emmy Noether). Finally, the great achievements of the axiomatic method in analysis are traced, although these considerations are tinged with an element of doubt as to whether the axiomatic change in analysis will ever be as complete as that in algebra.

No serious mathematician can afford to neglect these volumes. To read them is an experience; and, at the end, the reader will be more aware of the abiding serenity of his subject. **L. S. Goddard**

The Heritage of Copernicus: Theories "Pleasing to the Mind". (The Copernican Volume of the National Academy of Sciences.) Edited by Jerzy Neyman. Pp. 542. (MIT Press: Cambridge, Massachusetts, and London, 1974.) n.p.

THIS book is not about Copernicus, nor about the postulated revolution to which he has given his name. Rather, it is a collection of 24 essays, by various hands, dealing with various other postulated 'Copernican' or 'Quasi-Copernican' 'revolutions' in astronomy, cosmology, biology, chemistry, physics, mathematics, probability theory and technology. The book is intended for the general reader, who will need to have a rather large technical vocabulary, or access to a rather large and up-to-date dictionary. None of the contributors seems to be a professional historian of science.

Many of the 'revolutions' described are merely good pieces of traditional science—such as Shapley's work on our Galaxy—and almost all the references to Copernicus' own work show a degree of incomprehension that makes the book highly ambiguous as a tribute.

The most interesting essays are those which pay least attention to Copernicus or to 'revolutions': one by Geoffrey and Margaret Burbidge on some of the problems facing modern cosmological theories, and one by Stanley L. Miller on "The First Laboratory Synthesis of Organic Compounds under Primitive Earth Conditions".   **J. V. Field**

The Laue Method. By Jose Luis Amoros, Martin J. Buerger and Marisa Canut de Amoros. Pp. xi+375. (Academic : New York and London, March 1975.) $37.00; £17.75.

THE Laue technique, although the oldest used by X-ray diffractionists, was early superseded in many areas of structure analysis by methods based on monochromatic radiation and moving crystals. But it has remained a useful technique in metallurgy and crystal physics, especially, though no thorough presentation of its potentialities has appeared for about 40 years. This book aims to fill the gap.

Chapter 1 is historical, chapters 2–8 are written especially for the numerous users who have had no formal training in crystallography, and chapters 9–13 are aimed at the specialist. Structure analysts who normally only take Laue photographs by accident will find this book well worth reading, partly for the clarity of its presentation, but also because it presents some fresh and some neglected ideas. The novel suggestion for the use of the method in crystallochemical analysis seem to me to be rather too opti-

mistic, but the discussion of the idea that cylindrical Laue photographs can be used to determine the symmetry and orientation of an arbitrarily set crystal will well repay study. So too will the sections which discuss the methods used in the study of the diffuse scattering which results from thermal motion, disorder or crystal imperfections.

The last chapter purports to present a new method of interpreting the photographs; I can remember it being used, however, for teaching the topic at a summer school in 1947 and can attest to its utility.   **D. Rogers**

Books brief

Design Theory of Fluidic Components. By Joseph M. Kirshner and Silas Katz. Pp. xi+479. (Academic : New York and London, January 1975.) $45.00; £21.60.

AT present the list of books concerned with the relatively new technology of fluidics is quite short. Kirshner and Katz have made a valuable addition to the list. The initial chapters about the properties of fluid filled transmission lines and jet flows are particularly good and the bibliographies given after each chapter are extensive and well up to date. The authors have rightly restricted their attention to devices with non-moving parts and devote a complete chapter to each main type and their performance characteristics (these include the impact modulator, the vortex triode, the beam deflection amplifier, the bistable switch and the transition NOR). The appendices contain data on air filled transmission lines and also computer programs for calculating the response of such lines to various inputs. These will be very useful to system designers. Likewise, newcomers and students will find the list of problems following most chapters helpful, though for some reason the answers are not given. **Terry Hughes**

Mycotoxins. Edited by I. F. H. Purchase. Pp. xiii+443. (Elsevier Scientific: Amsterdam, Oxford and New York, 1974.) Dfl.115; $44.25.

THE discovery of aflatoxin 15 years ago attracted the interest of many scientists to the field of the mycotoxins. It was indeed fortunate as previously very little had been known of the chemistry and mode of action of the mycotoxins in spite of numerous ancient descriptions

of the massive poisonings of people and domestic animals by these fungal metabolites.

This book, addressed primarily to investigators and students, contains ample information on different aspects of studies on mycotoxins. Contributors have not followed precisely unified criteria in their presentations and some chapters are treated from widely different points of view. Furthermore, there are four chapters concerned with trichothecene mycotoxins, which results in some overlapping in the information on these compounds.

The authors have been chosen well. Most of them are leading authorities in the subjects which they write about, and the coverage is very good although there are few data dating from later than the end of 1972.

The presentation and typography of the book are excellent.

This volume should be useful not only to mycologists and scientists interested in mycotoxins but also to pathologists and experts in bromathology and toxicology. The book might be very encouraging to investigators interested in the mode of action of mycotoxins, since little work has been done on this aspect of these compounds.   **David Vazquez**

Handbook of Genetics. Vol. 1: *Bacteria, Bacteriophages and Fungi.* Edited by Robert G. King. Pp. xvi+676. (Plenum: New York and London, 1974.) $44.50.

THIS book, the first in a series, aims to review organisms which have been extensively used by geneticists. It is an excellent collection of review articles interspersed with techniques, gene symbols and chromosome maps for selected bacteria, bacteriophages and fungi. The introductory article on a classification and evolutionary scheme for all organisms, although interesting and relevant to the series as a whole, seems out of place as it makes no reference to techniques such as %G+C and numerical taxonomy. Most of the articles are of a high standard and contain a wealth of technical and background material. The total omission, however, of any reference to the extensive genetical work on *Coprinus lagopus* in an article entitled '*Coprinus*' is difficult to understand but this is an isolated case and the rest of the material is well balanced. The usefulness of the indices could have been improved by giving textual pages rather than bibliographical pages in the author index and by greater cross referencing in the subject index.

In spite of these relatively minor criticims and the high price, this book should become a basic reference work for research workers and teachers in microbial genetics.   **B. W. Bainbridge**

obituary

Robert Alexander Houstoun, a leader in the optics field, died on May 4 at the age of 92.

A student of Glasgow, he pursued his studies at Cambridge and Göttingen, where he obtained his PhD. Joining the staff of Glasgow's natural philosophy department in 1906 he continued his research in optics till he retired in 1948 and for some years after as an Honorary Research Fellow. His research interests were varied but he is best recalled for his work in colour vision and the measurement of the velocity of light in air and water by the piezo-quartz shutter method, which he developed. He also found time to write a considerable number of successful books. His *Treatise on Light,* first pub-lished in 1915, was for a long time the standard work in its field, and a considerable number of published papers flowed from his pen from 1904 to the end of the 1960s.

Eric H. Lenneberg, professor of psychology and neurobiology at Cornell University, has died at the age of 53.

At the time of his death, Dr Lenneberg was working on a clinical research project in neuropsychology at the Westchester Division of New York–Cornell Medical Center, after having joined Cornell in 1968. His major scientific achievement was considered to be the proposal, first advanced by him in the 1950s, that the human capacity for language could be explained only on the basis of the biological properties of the brain and vocal tract. He had summarised his experiments and views in his book *Biological Foundations of Language,* published in 1967. Dr Lenneberg was born in Germany and after spending much of his youth and childhood in Brazil, went to the US in 1945, eventually obtaining degrees from Columbia and Harvard. He joined the staff of Harvard, and later carried out basic research on language development in defective children at the Children's Hospital Medical Center in Boston while on the staff of the Massachusetts Institute of Technology. From 1967–68, he was professor of psychology at the University of Michigan.

announcements

Awards

The Royal Institute of Chemistry has made the following awards: **Meldola Medal and Prize** for 1974 to **P. J. Derrick** for contributions to the development of field ionisation kinetics and mass spectrometry; **Beilby Medal and Prize** for 1975 to **P. R. Swann** for work in electron microscopy of metals alloys and phase transformations.

Reports and publications

Great Britain

The Annual Report of the Science Policy Research Unit at the University of Sussex for the year 1974. Pp. 60. (Falmer, Brighton: SPRU, University of Sussex, 1975.) [15

Advisory Committee on Oil Pollution of the Sea. Annual Report for 1974. Pp. 28. (London: Advisory Committee on Oil Pollution of the Sea, 39 Victoria Street, 1975.) [15

Report by the Hydrographer of the Navy. Rear-Admiral G. P. D. Hall, for the year 1974. Pp. 40. (London: Ministry of Defence, 1975.) [55

Grassland Research Institute. Technical Report No. 16 March 1975. The Construction of Instruments for Measuring and Manipulating the Plant Environment. By J. E. Sheehy and A. M. Tearle. Pp. 36. (Hurley, Maidenhead, Berks: The Grassland Research Institute, 1975.) £1.50. [55

Royal Greenwich Observatory. Greenwich Time Report, Time and Latitude service 1974 January-June. Pp. 36. (Herstmonceux: Royal Greenwich Observatory, 1975.) [85

Oxford Biology Readers 78. The Productivity of the Sea. By D. H. Cushing. Pp. 16. (London: Oxford University Press, 1975.) 30p. [95

Institution of Gas Engineers. Communications. No. 955: Fifth Report of the Education and Training Committee, 1974/75. Pp. 26. £1. No. 956: Presidential Address by A. G. Pratt. Pp. 35. £1. No. 957: Technical Aspects of Natural Gas in Victoria. By J. M. Shaw. Pp. 24. £1. No. 958: Developments in Industrial Service in the West Midlands. By K. Manuel and J. W. Price. Pp. 26. £1. No. 959: Segas—a Suitable Case for Modelling. By H. W. D. Hughes and R. J. Martin. P. 26. No. 960: Gas from Coal for Synthesis of Hydrocarbons. By J. C. Hoogendoorn. Pp. 13. £1. No. 961: The Development of a Computer Assisted Customer Service System. By H. N. Coates and A. L. Hardy. Pp. 21. £1. No. 962: The North Sea and the British Energy Scene. By J. G. Liverman. Pp. 14. £1. No. 963: Money—at What Price? By J. H. Smith. Pp. 32. £1. No. 964: The SBGI and the Gas Appliance Industry—Its Role in Britain and Europe. By A. J. Adam. Pp. 17. £1. No. 965: Engineering for Expansion. By G. F. E. Roberts. Pp. 28. £1. (London: Institution of Gas Engineers, 1975.) [125

Meteorites: a Concise Account. By A. A. Moss. Pp. v + 26. (London: British Museum (Natural History), 1975.) 35p. [125

Structural Analysis and Structural Failure. By Professor E. H. Brown. (Inaugural Lecture. 13 November 1973.) Pp. 183-198. (London: Imperial College of Science and Technology, University of London, 1975.) £1.50. [125

Proceedings of the Royal Society of London. B: Biological Sciences, Vol. 189, No. 1095: A Discussion on the Recognition of Alien Life. Organized by N. W. Pirie, FRS. Pp. 137-274. (London: Royal Society, 1975.) UK £3.20; Overseas £3.30. [125

Some Results of Research in Chemical Engineering and Technology Supported by the Science Research Council. Pp. 15. (London: Science Research Council, 1975.) [145

Multiple Sclerosis. Pp. 40. (London: Office of Health Economics. 162 Regent Street, 1975.) 25p. [155

Philosophical Transactions of the Royal Society of London. B: Biological Sciences. Vol. 270, No. 907: The Behavioural Final Common Path. By D. J. McFarland and R. M. Sibly. Pp. 265-298. (London: The Royal Society, 1975.) UK £1.20; Overseas £1.25. [155

Competition, Risk and Profit in the Pharmaceutical Industry. (An Economic Review commissioned from Runnymede Research Limited and based on Studies by George and Priscilla Polanyi.) Pp. 53. (London: The Association of the British Pharmaceutical Industry, 162 Regent Street, 1975.) [165

Radiation Science at the National Physical Laboratory, 1912-1955. By E. E. Smith. Pp. v + 114. (London: HMSO, 1975.) £5 net. [195

Ireland—Oil and Gas Exploration. By K. W. Robinson and R. P. Riddihough. (Information Circular No. 8.) Pp. 11. (Dublin: Department of Industry and Commerce, The Geological Survey of Ireland, 1975.) [195

Journal of Antimicrobial Chemotherapy, Vol. 1, No. 1, March 1975. Pp. 1-129. Published quarterly. Vol. 1 (4 issues) Inland £9.50; Abroad £10.85. USA, Canada, Central and South America $24.50 plus $1.50 postage. (London and New York: Academic Press, 1975.) [195

Proceedings of the Royal Society of London. B: Biological Sciences. Vol. 189, No. 1096: A Discussion on Organic Pollutants in the Sea—Their Origin, Distribution, Degradation and Ultimate Fate. Organized by H. A. Cole and J. E. Smith. Pp. 275-483. (London: The Royal Society, 1975.) UK £3.20; Overseas £3.30. [205

Other countries

Institut Royal Météorologique de Belgique. Publications, Série B. No. 78: Sur la Simulation en Laboratoire des Mouvements Troposphériques de Grande Échelle. Par A. Quinet. Pp. 28. No. 80: An Iterative-Synoptic Method for Estimating the Barometric-Effect on Neutron Monitor Data. By Dr. A. O. Van Gysegem. Pp. 11. Publications, Série A, No. 87: Ozone and Total Oxidant Level in the Surface Layer at Uccle. By D.De Muer. Pp. 25. (Uccle-Bruxelles: Institut Royal Mét orologique de Belgique, 1975.) [304

Przeglad Bibliograficzny Roslin Leczniczych/A Bibliographical Review of Research on Medicinal Plants. Pp. 161. (Poznan: Instytut Przemysly Zielarskiego, 1974.) [304

University of California. Institute of Marine Resources. Biennial Report for the two years ending 30 June 1974. Pp. 50. (La Jolla: Institute of Marine Resources, University of California, 1974.) [55

National Research Council of Canada. Associate Committee on Scientific Criteria For Environmental Quality. Status Report. Pp. 63. (Ottawa: Publications, NRCC, 1975.) $1.25 [55

Smithsonian Contributions to Zoology, No. 177: Revision of the Blenniid Fish Genus *Omobranchus* with Descriptions of Three New Species and Notes on Other Species of the Tribe Omobranchini. By Victor G. Springer and Martin F. Gomon. Pp. iii + 133. (Washington: D.C.: Smithsonian Institution Press, 1975.) For Sale by US Government Printing Office. $2.65. [65

World Health Organization, Public Health Papers, No. 59. Administration of Environmental Health Programmes: A systems view. By Morris Schaefer. Pp. 242. (Geneva: World Health Organization, 1974.) Sw. Fr. 12. [65

World Meteorological Organization, Catalogue of Publications, 1951-1975. Pp. vi + 119. (Geneva: World Meteorological Organization, 1975.) n.p. [65

Publications of the United States Naval Observatory. Second Series Vol 22 Part 6. Photographic Measures of Double Stars. By F. J. Josties *et al.* (Washington, D.C.: US Government Printing Office, 1974.) n.p. [75

Environment Canada, Fisheries and Marine Service Technical Report No. 531. Harvesting, Processing and Composition of Sea Cucumber Meal as a Possible Substitution for Herring Meal in Trout Diets. By A. M. Suterlin and Susan Waddy. Pp. ii + 8. (St. Andrews, New Brunswick: Research and Development Directorate, 1975.) n.p. [75

Environment Canada Fisheries and Marine Service Technical Report No. 532. Observations on Lobsters and Scallops Near Pictou, N.S. By D. J. Scarratt. (St. Andrews, N.B.: Research and Development Directorate, 1975.) n.p. [75

Forever amber on manipulating DNA molecules?

LAST weekend saw a hundred scientists descending on Oxford to discuss with representatives of the Research Councils and the Department of Health and Social Security the problems of experimentation in the 'genetic engineering' field. It is now six months since the Ashby report was published and gave what we described at the time as an amber light to such experiments. The Ashby working party took evidence from more than twenty workers or potential workers in this field, but was itself constituted of scientists who in the main did not include those using the techniques and who might therefore have had a direct interest in the conclusions. Ashby reported that "subject to rigorous safeguards [spelt out in the report] these techniques should continue to be used because of the great benefits to which they may lead". But how to implement these safeguards, and how to implement them quickly?

Several participants were at pains to point out the intellectual pressure that is building up to get moving again in this field. It was wrily conceded that more impatient scientists were already under way and although there was no evidence that they were conducting experiments in an irresponsible way, their action was causing considerable frustration amongst those waiting for more formal guidance on safety. It is, as one participant put it, rather like the vivisection laws—until society has spoken it is left to the vagaries of individual consciences. And there is obvious pressure to go ahead—participants, on being asked whether they, as individuals, would wish to perform such experiments within the next two years, said 'yes' in considerable numbers.

If the issue were as simple as that, then the most obvious course would be to place as much pressure as possible on the Department of Education and Science, with whom the Ashby report now resides, to get cracking and persuade the government, amidst all its obsessions with consensus in inflation-control to give a moment's thought to the consensus in hazard-control, and implement the safeguards. (The meeting overwhelmingly endorsed the Ashby recommendations on strict containment.) But there are complicating factors.

First, the consensus amongst scientists that all could be made and kept safe is no longer accepted as the watertight guarantee that it once would have been. There has been a certain amount of self-satisfaction that where-

as the Asilomar conference was a meeting of those with a direct interest in the experiments, the Ashby report came from those who could take a broader view. But broad enough? Technicians have an obvious interest in safety, and their concern, expressed through their union, will not necessarily mesh with the scientists' desire to get moving as soon as possible. The pharmaceutical and agricultural industries, barely represented in the Ashby testimony, may at some time in the future wish to switch from laboratory to large-scale operations; they have an obvious interest in ensuring that the safeguards in any legislation make sense on the larger scale. And what about members of the public? Ashby went out of its way to ask 'how can the social values of the community at large be incorporated into decisions on science-policy?', and tried to present the report in a widely-accessible form. But how can the government assess what the community feels on this subject?

Second, there is doubt in some scientists' minds about the scope of any proposed code of practice. There was clear agreement at Oxford that Ashby and Godber should not be confused—that is, that there should be a difference in governmental approach towards the as yet unpredictable hazards of artificial recombinant DNA molecules and the predictable hazards of pathogens with their established handling procedures. But how open-ended should the discussion on unpredictable hazards be left? Some believe that the dangers in currently-known methods may seem slight in comparison with the dangers in methods only dimly foreseen. On the other hand, to widen the debate, the administrators in particular believe, would be to diminish the chances of a quick response from the government.

It may not be an issue of great substance but we feel that the exclusion of the scientific press from the Oxford meeting was unfortunate. We hardly believe that any dark conspiracies were hatched, but there is broader interest amongst scientists in this issue than in almost any other at present, and since one of the purposes of the meeting was to inform and educate, it should be said that an informed and educated press ought to have been one of the (admittedly lesser) aims of the organisers. It is difficult to accept the argument that participants would speak less freely with the press on hand, when there were already a hundred pairs of ears around. □

Man and the Biosphere

Tunisia: MAB project area No. 3 (grazing lands) examines the problems of rangelands supporting three million 'sheep units'.

When a conscientious government department requests advice on the potential environmental effects of a proposed agricultural development scheme for an area of tropical forest or of the construction of an irrigation dam in an arid area, it is a chastening experience for the "scientist" to have to admit that he has no hard and fast advice to give. Contrary to what most people believe, we neither know for sure what should be done nor, equally important perhaps, what should not be done in these kinds of situations. For, paradoxically, although a great wealth of scientific information is available, much of it is not used and, in fact, in the mono-disciplinary form in which it has been compiled, is unusable within the context of development planning. In spite of the years of work that have gone into the study of forests, deltas and estuaries, arid zone grazing land and irrigation problems, and so on, the findings that have emerged, valuable as they are, do not provide practical answers to the sort of questions governments and decision-makers are asking, because the research approach has not been geared to consideration of the man/environment system involved. It was precisely to resolve this paradox that UNESCO's Man and the Biosphere (MAB) Programme was conceived in an attempt to develop an integrated research approach to the management problems arising from the interactions between human activities and natural systems. Michel Batisse, Director, Department of Environmental Sciences and Natural Resources Research, UNESCO, reports.

THE response to MAB at government level, particularly from the developing countries, was favourable from the start, yet it requires a very long time and much patient effort to get any project moving on a reasonably harmonious front at the international level. The reasons for this inertia are easy to understand. They relate to the traditional suspicion in the older countries of anything 'supranational', to the difficulties of fully conveying the concepts underlying a common objective in a multilingual and multicultural world, and to the mere fact that not all countries are, to put it mildly, at the same stage of development and at the same level of capability. For those with no experience of the intricacies of international cooperation, this slow movement is thoroughly frustrating. For those aware of the problems, it is always a pleasant surprise when some progress is actually made.

The MAB Programme provides a good illustration of this. It originated from the conference in September 1968 on "the scientific basis for rational use and conservation of the resources of the biosphere", which could hardly have dealt with a subject of greater concern to all men and countries. Yet it took some two years before the programme could be actually formulated and officially launched—in November 1970—by the General Conference of UNESCO. And it took another two years of misunderstandings and foot-dragging before it was unanimously endorsed by the Stockholm Conference on the human environment. What had been initiated through the enthusiasm of working scientists had to find its way through the tortuous pathways of politics and the minefields of official science before it could be presented to the world with all the necessary visas and blessings.

This has now been done, and another major step forward has since been achieved; last September, in Washington, DC, the International Coordinating Council which supervises the Programme recognised that the preparatory phase was over and that MAB could now enter its operational phase.

Although MAB was discussed during the summit talks between the USA and the USSR last year—to the great bewilderment of the chancelleries when they discovered this strange acronym in the official communiqué—little publicity has so far been given to the Programme in the scientific press and its actual scientific content is not known to many people potentially interested in it. The general objective has been formulated as follows: "to develop within the natural and social sciences a basis for the rational use and conservation of the resources of the biosphere and for the improvement of the relationship be-

tween man and the environment; to predict the consequences of today's actions on tomorrow's world and thereby to increase man's ability to manage efficiently the natural resources of the biosphere".

To achieve this ambitious objective MAB has adopted an integrated, global approach to the analysis of ecological systems, their structure and functioning and their mode of reaction when exposed to human intervention. This approach stresses the impact of man on the environment but also the impact of the environment on man.

The MAB Programme comprises fourteen project areas forming a kind of research matrix in which the main ecological systems and physiographical units (tropical forests, grazing lands, mountains, islands, river basins and estuaries, and so on) interact with major activities or processes such as conservation of genetic resources, use of pesticides and fertilisers, major engineering works or demographic changes.

The MAB Programme is intergovernmental in structure: it is controlled by an International Coordinating Council consisting of representatives of 25 member countries, sitting together with representatives of the organisations of the UN system interested in the Programme (UNEP, the FAO, the WHO, the WMO and UNESCO) and representatives of ICSU and the IUCN. The contribution of each country to the international programme is developed through National Committees, where normally scientists from universities and research institutions sit alongside representatives of government departments. The general secretariat is provided by UNESCO.

Today, after the somewhat tedious but all-important period of preparation during which working groups and expert panels hammered out research guidelines for all projects, the stage is set for actual implementation of cooperative programmes at the regional and international level. Some 80 MAB National Committees have been established, not all of them perfect but most of them keen to move ahead.

Underlying the Programme are three concepts on which its approach to research is based: MAB is problem-oriented, it is interdisciplinary and it aims to provide both immediate and long term scientific, economic and social benefits, in particular to the developing countries.

A problem-oriented approach to research has the twin advantages of projecting the practical face of science to government leaders and of involving decision-makers in the formulation and implementation of projects. Within MAB, the scientist who has traditionally been concerned with the question

"what happens?" in an ecosystem is asked to go a step further and attempt to answer the question on which the decision maker will base his judgement, that is, "what would happen if . . . ?"

Work now being undertaken in Tunisia within the framework of MAB Project Area No. 3 (grazing lands) provides a good example of this problem-oriented approach. The arid and semi-arid areas of southern and central Tunisia cover 65,000 square kilometres and account for some 40% of the national territory. Intense pressure is being exerted on this area by the 3 million "sheep units" it harbours (the various categories of domestic animals being statistically accounted for in terms of sheep equivalents) and by a human population of 1 million urban and 2 million rural inhabitants. The sort of information Tunisian planners want is: what is the carrying capacity of the rangelands and how can it be improved? How far should the development of intensive farming be improved? How far should the development of intensive farming be encouraged? What would be the ecological effects of the introduction of improved fodder crops? The answers provided will have a major bearing on the ecological, economic and social future of the country, and will be of obvious interest to other countries in the Mediterranean area having similar conditions.

The interdisciplinary approach is, by the very nature of things, essential to the MAB programme, but there are considerable difficulties in creating interdisciplinary research teams and in integrating fully the results achieved.

It is easy enough to talk about the need to break down the barriers between disciplines, but many of these divisions have a logical and practical basis. The units of analysis employed often differ from one discipline to another. Socio-cultural studies are usually based on social units—kin groups, tribes, communities—whereas ecological studies may be based on sample areas of a few hectares or even less. Problems of the duration of research also arise. A botanist may be interested in the problem of biomass changes in a plant community during a single growing season, whereas the sociologist may need to examine migration trends over a number of years. If an interdisciplinary effort is to succeed it must be planned as such from the start; each research worker must be prepared to adapt to the methods of others and all must possess the flexibility of mind and the team spirit that this approach implies.

The vital importance of interdisciplinarity is well exemplified in two current MAB projects. In the study on population and environment in the eastern

islands of Fiji (MAB Project Area No. 7—island ecosystems) the research team consists of ecologists, pedologists, a nutrition expert, human geographers, a biogeographer and marine biologists. The objective is to understand the actual relationships between population levels and natural resource use under well established conditions. In Hong Kong, where an integrated ecological study of a large urban settlement is being carried out with Australian support (MAB Project Area No. 11—urban systems), the spread of disciplines is even wider; the fifty-strong research team ranges from biologists and hydrologists to sanitary engineers, architects and town planners, physicians and psychologists, cultural anthropologists and sociologists. The team also includes a small unit which specialises in the study of the problems of research integration. The objective is, of course, to understand the functioning of the urban system as a particular kind of ecosystem, in order to provide a sounder basis for its planning and management.

It is interesting to note that—in contrast with most previous international research efforts—the MAB Programme stimulates a particularly strong interest in the Third World countries. Determined to shake off the vestiges and constraints of their past, they are eager to exploit their natural resources for their direct benefit. But they are more aware than one thinks of the difficulties involved in changing traditional methods of land use or introducing foreign technologies. They seem therefore to have quickly realised that MAB can offer them tools with which to formulate important aspects of their development planning. A typical example is the problem of the tropical forests. About half the world's forest area is located in three main regions: tropical Africa, tropical America and South-east Asia. Tropical forests are estimated to cover an area of some 2,000 milion hectares, of which some 850 million hectares can be regarded as tropical rain forests. Forests are seen by developing countries of the tropics as largely unexploited areas for colonisation and agricultural development as well as a potential source of foreign exchange. For the developed countries they represent a readily available source of relatively cheap timber.

Widespread modification or destruction of tropical forests is of concern to scientists because of their ecological diversity, complexity of structure and richness in species. Within the tropical region, intimate relationships exist between the indigenous populations and the forest, and the forest is important for soil and water conservation. Because of their extent, biomass and dynamics, tropical forests also play an important yet little understood role in the global

ecological and atmospheric balance of the biosphere.

The importance of research in this field was recognised from the inception of MAB and a general formulation of the practical research problems involved was made in November 1971 under MAB Project Area No. 1 (tropical and sub-tropical forest ecosystems). The next step was to convene in May 1972 an *ad hoc* panel of experts to elaborate the scientific content of research in this area and to consider approaches and plans of studies that could be recommended to the MAB National Committees concerned. This was followed up by an international working group meeting held in Rio de Janeiro in February 1974 which was given the task of identifying problems of common concern and of examining research proposals presented by the different governments that would involve international cooperation. The working group was also asked to discuss the requirements for regional coordination of research, harmonisation of methods and measurements, the exchange of information, experience and personnel, the training of specialists and technicians, and the sharing, storage and processing of data. Finally, regional meetings have been organised in Kuala Lumpur and Mexico City at which national representatives proposed specific pilot field projects for the regions concerned. Similar regional meetings are to be held in Kinshasa and Varanasi later this year.

At the same time regional long term training courses on tropical ecology have been organised in Venezuela, the Philippines and Kenya to contribute to the training of ecologists who could be used in the development of research and management programmes in the three regions concerned. Finally, a world-wide synthesis of knowledge on the tropical forest ecosystems and their rational use and conservation is being prepared for publication in 1976 so as to provide a common background and conceptual basis for MAB Project No. 1.

As a result of this strategy, one can envisage within the near future the development of a network of pilot schemes for integrated ecological research in the three tropical forest regions of the world, involving the exchange of research personnel, providing field training, and forming part of an inter-regional co-ordinated effort to help master one of the most challenging problems of the tropical world. A similar 'strategy' is being applied for other MAB project areas, particularly those on grazing lands and urban systems.

Is MAB now really going to take off? At the last session of the UNESCO General Conference, where some 130
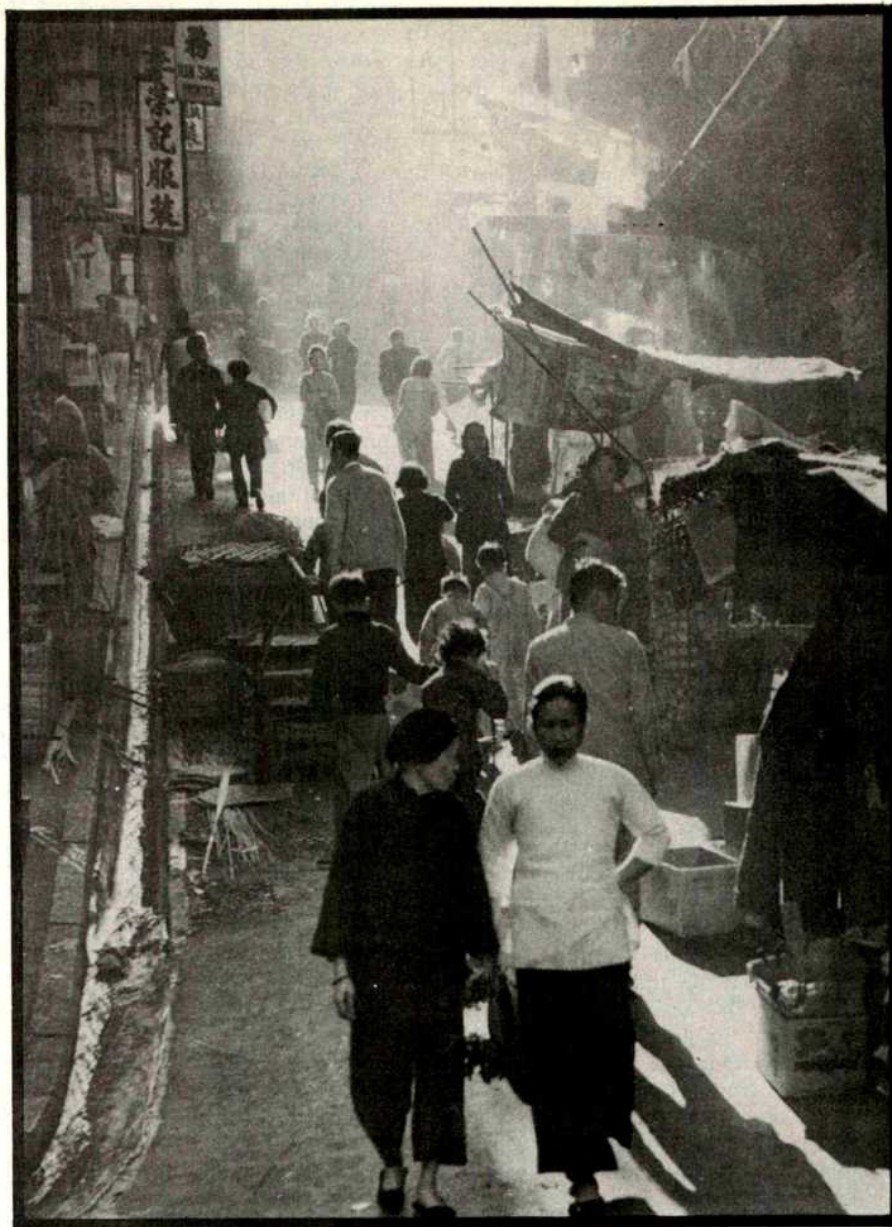
Hong Kong: chosen by MAB for an integrated ecological study.

governments represented were sharply divided on several issues, MAB received unanimous support. The political obstacles therefore seem minimal. Governments, particularly those of the developing countries, are very interested and those scientists already involved in the Programme are committed to its success. The major difficulties are probably elsewhere. They lie primarily in the difficulties—lack of scientific infrastructure and of personnel—developing countries face in playing their role in a world-wide programme, which would be meaningless without their participation. This can only be mitigated through a vigorous training and technical assistance back-up effort in which all donor countries and international organisations concerned should give a hand.

The role already played by UNEP in support of MAB has already been significant, particularly for the regional

activities which are undertaken.

But ultimately the key to the whole operation probably lies in the hands of scientists from the industrialised countries. For them, the programme offers access to areas of the world in which challenging research projects are being developed, either through direct co-operation between MAB National Committees or through multilateral channels. It offers also the interest of adapting to new interdisciplinary methods and to a spectrum of research, the social value of which is beyond question.

Times are difficult everywhere for research funding, but the case for an environmental research programme such as MAB is unanswerable. The scientific community could, if it so wished, demand and obtain the necessary facilities which it alone can mobilise and utilise to make MAB a true and lasting success. □

international news

PRIME Minister Pierre Trudeau has defended Canada's sales of CANDU nuclear reactors to developing countries on the grounds that she cannot withhold from them the advantages of nuclear technology. In an address to the annual meeting of the Canadian Nuclear Association (CNA), he said: "We are a society which has not forgotten its frontier origins. We are a people who have experienced the torment of need, who understand the benefits of sharing. It is inconsistent with that experience and that understanding that we should now deny to the less developed countries of the world the opportunity to gain a handhold on the technological age . . .

"Surely, if we are ever to eliminate the immense disparities which now separate the living standards of rich and poor, it will be necessary to make available to the disadvantaged every technique at our disposal."

It would be unconscionable to deny to the developing countries the most modern of technologies as assistance in their quest for higher living standards, the Prime Minister said. "But in a world increasingly concerned about depleting reserves of fossil fuels, about food shortages, and about the need to reduce illness, it would be irresponsible as well to withhold the advantages of the nuclear age—of power reactors, agricultural isotopes, cobalt beam therapy units."

As a result of the explosion by India of an underground nuclear device last year, which was made with material from a reactor supplied by Canada, the Canadian government has come under criticism from both within the country and without. Critics have suggested that such an event should have been foreseen by Canada, and prevented.

Since the Indian explosion, the federal Cabinet has done some soul-searching and has tightened its nuclear safeguards policy—but it has also confirmed Canada's intention to go on selling nuclear reactors abroad. This decision, too, has drawn criticism.

In an obvious reference to such criticism, Mr Trudeau said: "We can be proud, as Canadians, of our cooperation with India. The decision taken by Prime Minister St Laurent to enter a nuclear assistance programme with India was a far-sighted and generous act of statesmanship."

Nuclear 'transfers' should always be

Canada answers her nuclear critics

from David Spurgeon, Ottawa

subject to safeguards, however, said the Prime Minister. Canada has now raised the standards of her safeguards "to the point that they are the toughest in the world [and] we refuse to engage in nuclear cooperation without an explicit exclusion of explosive uses."

Mr Trudeau said he did not pretend that the present system of inspection and detection of nuclear cheating was foolproof, and he was "painfully aware that the NPT [Non-Proliferation Treaty] is yet far from universally supported." But he reminded critics that the statute of the International Atomic Energy Agency (of which Canada is a member) charges the agency to spread peaceful applications of nuclear energy throughout the world, "bearing in mind the special needs of the under-developed areas."

(The latest element of the Canadian safeguards policy was announced May 4, 1975, by the Secretary of State for External Affairs at the NPT review Conference in Geneva. It provides that future Canadian bilateral nuclear commitments will be undertaken solely with countries that are parties to the NPT. Also, ratification of the treaty will be an important factor in Canada's decisions on provision of government financing in the nuclear field.)

The existence of some adverse public opinion concerning nuclear matters in Canada was acknowledged at the conference at which the Prime Minister spoke. The CNA's public relations committee said in its report:

"A number of new groups opposed to nuclear power developments have formed in Canada in the past year and there have been modest attempts to form a federation of such groups across the country, which suggests a new phase in the industry's relations with the public may be beginning."

The association's President, J. M. Douglas, President of Babcock and Wilcox Canada Limited, said that although the Canadian government and nuclear industry believe the CANDU system can give great benefit to

Canada through domestic and export markets, "if the opinion of a good majority of Canadians is not in accord with that of government and the utilities, our country's position with regard to the generation of electrical power in a few years' time could be put in jeopardy—as is happening in some other countries."

This seems to be happening because positive, factual information has not been made available in a clearly understood form, Mr Douglas said. "Small, but well organised, environmental groups have used information which is incomplete or inaccurate or misleading, to produce scare stories."

Too often this information—in the absence of factual information supplied from an informed source—has influenced fair-minded people against nuclear power, he said. "We must not let this happen in Canada. And it could. We must greatly increase our efforts to communicate factual information to those who seek it . . ."

Plans already have been made to do this, with a seminar to assist member organisations to communicate with various publics, and a new booklet.

In a review paper, J. S. Foster, President of Atomic Energy of Canada Limited (AECL) said Canada today has 2,500 MWe of nuclear electric generating capacity in operation and a further 6,000 MWe under construction. An additional 7,000 MWe is either committed or at an advanced stage of planning. By the end of the century there will be about 130,000 MWe produced by nuclear plants in Canada.

● L. J. Schofield, of the CNA's Economic Development Committee, says that there is a significant advantage for nuclear power generation in Canada if coal prices escalate at 8% or more a year; such rises would be offset only by sustained capital cost escalation rates in excess of 13% a year. At low coal price escalation rates of 5%, however, the competitive advantage of CANDU stations is small, and could be completely offset by double-digit inflation of capital costs.

"Double-digit inflation," says Schofield, "is a factor to be reckoned with today by the Canadian nuclear industry . . . If the costs of fossil and nuclear stations inflate at equal rates, then the advantage of nuclear generation due to lower fuel costs could be progressively offset by the increasing differential in capital cost." ☐

Soviet meetings, great and small

from Vera Rich

ONE of the most striking features of Soviet science is the large number of scientific conferences to which it gives rise. Leaving aside, at the one end of the scale, the major conferences of international bodies which happen to have chosen a venue in the Soviet Union and, at the other, small and relatively local gatherings, there still remain 25–30 All-Union meetings, conferences, symposia, and seminars which take place each week. In all, some 1,400 such conferences are scheduled for 1975, with an estimated attendance of some 210,000 scientists and technologists.

Although such conferences are conventionally hymned by Soviet planners as forming one of the most fruitful means of implementing the results of basic research in the national economy and of promoting interdisciplinary studies, in reality not all is well with the Soviet conference scene. According to a recent *Pravda* editorial, many conferences are becoming afflicted with the disease of "gigantomania". With several thousand scheduled speakers, a situation is reached where, even with drastic subdivision into different working groups (which, to a large extent, limits interdisciplinary contacts to outside the lecture hall), each lecturer is allotted only a few minutes in which to deliver only an abstract of his paper. This would be slightly less serious if the audience had already read it in preprint form, so that the short time available could be devoted to questions. But this, too, is not possible—gigantomania besets the preprints too. At the Fourth All-Union Seminar on "Methods and instruments for measuring discharges and quantities of gas" only one-fifth of the papers were ready in preprint form. The post-conference publication of proceedings is likewise extremely tardy so that the papers have lost much of their value before they ever appear in print. This problem, of course, is not peculiar to the Soviet Union—in few other countries, however, would it be considered so urgent as to merit editorial coment in the country's leading newspaper, recommending that steps should be taken to rectify the situation by all the bodies responsible, from the Academy of Sciences and the relevant ministries downwards.

● The overlap between the two forms of Soviet scientific meetings—the vast gigantomaniac official gathering and the small illicit gathering of refusniks —sometimes approach each other in a manner somewhat perturbing to the

authorities. This, according to his sons, is one of the main reasons why Academician Veniamin Levich has received such opposition to his desire to emigrate to Israel. So far, he is the only Member of the Academy of Sciences to request a visa (Professor Tumermann was, at the time he applied for a visa working at the Lebedev Physics Institute of the Academy but

Academician Levich: promised a visa.

was not himself an Academician). Levich who has long since been dismissed from his post still remains a member of the Academy, and, technically speaking, still retains the right to attend meetings. Although Levich's works are no longer mentioned by establishment Soviet scientists, and references to them in incoming Western works are blotted out by the censor, the authorities seem to prefer to let him remain an Academician, rather than put into action the complex machinary of expulsion, which could well bring a backlash of unfavourable publicity. Academician Levich has been promised his exit visa—"by the end of 1975". With the 250th anniversary celebrations of the Academy scheduled for October, it will be interesting to see whether Academician Levich will receive his visa before that date—or if not, whether he will assert his right to participate in the jubilee meeting.

● Another interesting problem is that of refusnik scientists invited to take part in international conferences to be held within the Soviet Union. The case of cyberneticists Aleksandr Lerner and Evei Gel'man is likely to provoke a problem for the Soviet authorities.

Lerner and Gel'man have been invited by the international organising committee to take part in the Conference on Artificial Intelligence to be held in Tbilisi this September. Judging from the case of Zhores Medvedev, who was forcibly prevented from attending the Kiev Conference on genetics, it seems unlikely that Lerner and Gel'man will be permitted to attend the Tbilisi Conference. How the problem will be resolved is still a matter for speculation, but latest reports indicate that Gel'man has been promised an exit visa, although he has not, to date, actually received it. To send both of them abroad before the Conference would certainly prove the happiest solution for the two refusniks themselves, and could well be the easiest way out for the Soviet authorities of what might otherwise prove an embarrassing and publicity provoking situation.

● A conference liable to cause a certain unease to the Soviet authorities was that of the Royal College of Psychiatrists, which held its annual meeting in London last week. The meeting unanimously confirmed a decision to send a telegram of protest to Professor A. V. Snezhnevskii, President of the Institute of Psychiatry of the USSR, deploring the misuse of psychiatry as a means of political repression. The telegram, signed by Sir Martin Roth, president of the college, cites specifically, "the continued incarceratian of Gluzman, Bukovskii and Blyushch, which appears a perversion of psychiatric practice and the denial of natural justice".

The case of Dr Gluzman, whose incarceration in a mental hospital seems to have been the direct result of his preparation of a report on psychiatric malpractice in the case of General Grigorenko, was raised at last year's Annual Meeting. Since then Sir Martin Roth has sent two letters to Professor Snezhnevskii on Gluzman's behalf, the first of which received an extremely unsatisfactory answer, stating that the case against Dr Gluzman contained no references to the Grigorenko affair. The second letter, which in effect was an enquiry as to what then were the charges against him, received no answer.

The telegram of protest also cites the case of Vladimir Bukovskii, co-author with Dr Gluzman of the famous *samizdat* "Manual of Psychiatry for Dissidents" (a guide to what to do if accused of mental illness as a result of one's political views) and Leonid Plyushch, the Ukrainian mathematical, who, as a result of the "treatment" he has undergone since hospitalisation for dissidence is now, according to the latest report (from an informant who saw him last April) in a state of virtual

catatonia with probably irreversible mental damage.

In addition to the protest to Professor Snezhnevskii, the meeting also resolved to send a telegram to Dr Gluzman himself—the first time that this particular form of protest has ever been undertaken by the college.

Among those who spoke in favour of this action was Dr Denis Leigh, Secretary General of the World Psychiatric Association, who said that in his view the type of action envisaged by the college was welcome and much to be encouraged. □

Oxford man wins large prize

PROFESSOR X has won for Dr Y from Oxford (who prefers to remain anonymous) a year's free subscription to *Nature*. Professor X is on 12 editorial boards and easily heads the list in *Nature's* competition (May 8); the runner-up could muster a mere nine appearances. □

THE effects of inflation on the prices of technical and scientific books are illustrated in this table of findings from a British survey. Eleven publishers submitted information for the survey, which was carried out by the Technical and Scientific Group of the Publishers Association Each was asked to give the extent and price, on publication, of their new scientific and technical books (and new editions) published in the years 1972, 1973 and 1974. Where there were two editions (eg, cased and paper) the cheaper was taken. In the case of 1974 they were asked to distinguish between publication in the first and second halves of the year. They were also asked to assign each book to a category, namely: A: books intended for purchase by undergraduate students; B: books intended for purchase by senior students, lecturers, practitioners, libraries, etc; C: books for which only a library sale is expected. The most rapidly rising cost was that of paper.

Year	Size of Sample	Total no. of pages	Total price (£)	Price/ 100pp (£)	% above 1972	
1972	157	44462	381.75	0.86	—	
1973	140	39062	390.15	1.00	16.2	Category A
1974(1)	74	21343	218.25	1.02	18.6	
1974(2)	49	12029	138.75	1.15	33.7	
1972	100	29297	457.95	1.56	—	
1973	95	28831	483.53	1.68	7.7	Category B
1974(1)	57	18511	333.95	1.80	15.4	
1974(2)	35	9657	179.70	1.86	19.2	
1972	31	12169	249.20	2.04	—	
1973	45	18842	397.00	2.11	3.4	Category C
1974(1)	23	8696	231.50	2.66	30.4	
1974(2)	10	4073	163.80	4.02	97.0	

correspondence

Deep in Egypt

SIR,—Having carried out surveys during and after the Second World War in Libya, Egypt and the Sudan, I was very interested to read the article (June 19) on the project for producing power by diverting Mediterranean water into the Qattara Depression by a canal through the Alamein area. Your correspondent was incorrect, however, in ascribing the initial surveys and ideas for this project to Professor Bassler, an ex-officer of Rommel's army; it is much older than that. In fact it dates back to the previous war, since it was in 1917 that Dr John Ball, the Director of the Egyptian Desert Survey, lent a small aneroid barometer to the officer in charge of a British Light Car Patrol who was going to travel in the area. The officer came back without the aneroid but with a reading for the Qattara Spring of some 60 metres below sea level—and presumably with a somewhat tarnished reputation as a surveyor!

Dr Ball did not, however, forget this odd result; and in 1926 he was able to send a professional surveyor, G. F. Walpole, to make a more detailed investigation, which revealed for the first time the full extent and depth of this remarkable feature. Describing this and other investigations in 1927 in the *Geographical Journal*, Dr Ball then suggested that the depression might be used as a source of power by diverting Mediterranean water into it through tunnels. Six years later, when a complete topographical and geological survey had been made, he published a long paper in the same journal, giving detailed estimates of the sizes of tunnels required, the energy available and the length of time (several hundred years) that the project could last. Clearly the rate at which salt will accumulate even from sea water is very much slower than the rising tide of silt in the upper reaches of Lake Nasser, even though with modern engineering techniques much larger channels or tunnels can be envisaged.

Dr Ball, like Dr H. E. Hurst who organised the comprehensive collection of data about the Nile Basin on which so many projects were based, belonged to a small band of British surveyors and scientists who worked for many years in Egypt in the early years of this century. With the wisdom gained from long experience in the area added to their native wit and scientific training, they wrote a number of far-sighted papers which those who are now suggesting new versions of their projects will do well to study.

Yours faithfully,
JOHN WRIGHT
Effingham, Surrey

English and editorial boards

SIR,—Mr Andrewes describes himself as a hack. Nothing in the wording of his letter (July 10) suggests that the epithet is justified, but there are many people around editorial offices to whom it is applicable. Instead of confining themselves to turning poor into passable English, they seem to delight in trying to turn straightforward English into jargon. In a proof that came last week an editor was trying to get me pompously to refer to myself as 'the author'. Proofs of two papers sent to a microbiological journal a few years ago had been so mangled that I withdrew them and published them as originally written in *Proc. R. Soc.* **B.** A friend had a recent paper to a nutritional journal turned in places into germanic near gibberish. And so it goes on, making people who recognise clear English think that all scientists write badly.

If a paper comes within the province of a journal and seems to be scientifically acceptable, an editor's job is the elimination of ambiguity and prolixity. These are matters of fact. An editor can say: "This phrase could mean A or B. Which do you mean?" or "This amounts to so-and-so, which is only half as long as the original." It is no part of an·editor's job to insert misconceptions about the English language, however sincerely felt, into someone else's paper.

Yours faithfully,
N. W. PIRIE
Harpenden, Herts,

Fostering spin-off

SIR,—It was with much interest that I read the recent paper by Isaacs *et al.* (January 24) on cyclogenesis by motor vehicle movements. I wonder how many people outside Australia are aware that the Aborigines have been making use of this principle for centuries. Why else would their rain making dances consist of a steady clockwise progression?

Related to this, I am at present working on a theory that the drying up of central Australia followed the invention of the boomerang, whose anticlockwise rotation would inhibit cyclonic activity. As happens with most revolutionary ideas, however, I have been having trouble funding this programme.

Yours faithfully,
K. H. LLOYD
Salisbury Heights, South Australia.
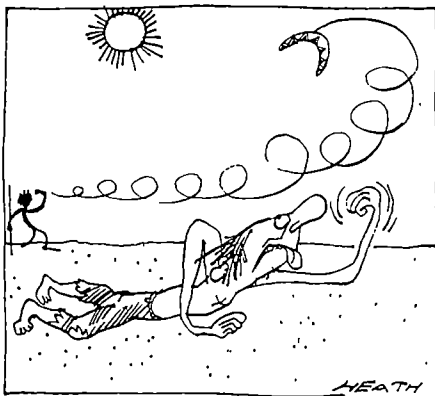
Darwin's cancer research

SIR,—In considering the scope of subject matter relevant to the study of cancer, Stoker (April 17) suggests that much of cell-oriented biology may have such implications. It may, but it would be shortsighted to stop there. Natural selection has obvious relevance, at least obvious to an evolutionary biologist, and Cairns (May 15) has discussed this explicitly. It has also become central to immunology. My only contribution to oncology came as a direct result of work in palaeoecology. Theoretical frameworks that develop in one subject often have application elsewhere, and this really cannot be foreseen. Would

the Imperial Cancer Research Fund have supported Darwin?

Yours faithfully,
LEIGH VAN VALEN
University of Chicago,
Chicago, Ill. 60637

Integrity in science

SIR,—I am sorry that the only comment accompanying my letter (June 12) mainly concerns its occasion and not its essence. I would gladly ignore it, but the claims of truth preclude that luxury.

Mr. Maddox's suggestion that the non-appearance of his leader resulted from the "offensiveness and repugnancy" of the correspondence is false. The article was promised, "before the end of the year", on November 24, 1969. Successive (polite) enquiries from Lord Soper and me brought such replies as "the article is now almost ready" (January 21, 1970); it would be delayed "a week or two" (end of March 1970); until Lord Soper's final inquiry (July 6, 1970) brought no reply—or the article. It had been promised in place of my reply to Professor Synge who had written (*Nature*, **219**, 790; 1968) that "as the result of a lengthy correspondence with Professor Dingle" he and I had agreed that either "the concepts used in the special theory of relativity as ordinarily understood" or "the concept of clocks that run regularly, as understood by Professor Dingle" must be abandoned. Since my concept of such clocks has never been represented as other than an instrument so recognised by a standard observatory, the only relevant property I required being an inability of one to run concurrently both faster and slower than another, it was now clearly mate in one move. Maddox made·that move impossible by steadfastly suppressing my rejoinder; it has never appeared. (Incidentally, his statement now that I have "shifted my ground" since 1968 is here seen clearly to be false: Synge's diagnosis is identical with my position as stated in my recent letter).

Maddox should have quoted more of my letter of April 6, 1971; it ran: "In view of the failure of all other means of getting a straightforward answer to my criticism of special relativity . . . I have been, with great reluctance, forced to the extreme measure of writing a book describing the course of the whole series of evasions . . . I write this final letter, which will be included in the book if necessary, to invite you to give your own explanation of your attitude, which I promise to include verbatim. I repeat what I have said before—that my sole object is to get this matter settled, first of all with absolute openness ·and avoidance of all further evasion and quibbling, and secondly,

provided that that is done, with the minimum of sensation and unpleasantness. Action on your part, even now, would make a full exposure of the ethical aspect of the matter unnecessary" Your readers can judge whether the "offensiveness and repugnancy" of this letter adequately excuse the succession of broken promises a year earlier.

There was a sequel to "the recent incident" as related by Mr. Maddox. The author of the article mentioned subsequently asked for his side of the story (although, since it had already appeared in the Editorial, this might well seem redundant) and was given a vague statement that implied, if anything, that the "promises" ascribed to me had another source than that which he now owns, which was "even more full of somewhat unrealistic threats". I know of no letter which can be so interpreted. It was to prevent a recurrance of such misrepresentation and for that reason alone that I felt it necessary to record the actual facts.

I suppose I must reply once more to the charge that "Dingle's confusion stems from his assertion that special relativity requires that the differences of rate should 'actually and not merely apparently' occur. The truth, of course, is quite the opposite." Whenever the special relativity effects of motion are invoked to predict or explain something observable (for example, asymmetrical ageing, cosmic-ray behaviour . . .) they are held to be "actual", whenever this leads to a contradiction they become only "apparent"; and anyone to whom this is unacceptable is deceived by "commonsense". The letter I have called "L" completely refutes Maddox's statement. His technical example involving "lasers" and "algorithms" refers to a completely different phenomenon which has nothing at all to do with the matter.

I withhold comment on the rest of Maddox's letter, remembering what Shakespeare said about painting the lily, and leave your readers to judge whether Maddox is right in denying that there is "an ethical issue" here. I am sure, however, that the many who agree with me that there is, will require a plain, direct answer to my question if they are to retain their trust in the integrity for which the scientific world has in the past been justly noted. I most earnestly hope that among those with authority and responsibility in this matter, there will not be wanting someone ready to have done with "doublethinking", to clear his mind and words of cant, and to exhibit the candour and courage needed to provide such an answer.

Yours faithfully,
HERBERT DINGLE
Purley, Surrey

news and views

Applying molecular genetics to a human disease

from Edward J. Benz and David G. Nathan

THE α- and β-thalassaemia syndromes are a heterogeneous group of inherited haemoglobinopathies characterised by reduced or absent synthesis of structurally normal α or β globin chains. The first step to identifying the nature of the syndromes was made in 1971 when the *in vivo* defect in chain synthesis was reproduced *in vitro* using β-thalassaemia globin mRNA (Nienhuis and Anderson, *J. clin. Invest.*, **50**, 2458; Benz and Forget, *J. clin. Invest.*, **50**, 2755). Then a quantitative deficiency in globin mRNA was demonstrated by the technique of hybridising thalassaemic globin mRNA with radioactive DNA complementary to globin mRNA (Housman *et al., Proc. natn. Acad. Sci. U.S.A.*, **70**, 1809; 1973; Kacian *et al., Proc. natn. Acad. Sci. U.S.A.*, **70**, 1886; 1973). Total absence of cytoplasmic β chain mRNA in homozygous β^0-thalassaemia (Forget *et al., Nature*, **247**, 379; 1974; Benz *et al., Blood*, **45**, 1; 1975) and α-chain mRNA in homozygous α^0-thalassaemia (Kan *et al., J. clin. Invest.*, **53**, 37a; 1974) has subsequently been demonstrated both by translation in cell-free systems and by complementary (c) DNA–mRNA hybridisation.

It is, therefore, now clear that the primary genetic lesion in thalassaemia is a defect in the synthesis, processing, transport, or stability of the α chain mRNA (in α-thalassaemia) or β-chain mRNA (in β-thalassaemia). Since total absence of mRNA in the α^0- or β^0-thalassaemias suggests gene deletion, investigators are now concentrating on the regulation of human globin gene expression. These disorders thus continue to be the examples *par excellence* of human diseases to which the techniques and concepts of molecular biology can fruitfully be applied.

Ottolenghi *et al., (Nature*, **251**, 389; 1974) and Taylor *et al., (Nature*, **251**, 392; 1974) recently isolated total cellular DNA from livers of infants with Hb Bart's hydrops foetalis, the homozygous form of α^0-thalassaemia. This DNA hybridised to purified β-chain cDNA as efficiently as normal DNA but failed to form any double stranded hybrids with α-chain cDNA. This constitutes strong evidence that these infants had no α chain genes

and that, therefore, the α^0-thalassaemia allele is a gene deletion.

Kan *et al. (Nature*, **255**, 255; 1975) have now applied the same approach to answer a more vexing question: the number of α chain genes present in normal man. There has been considerable controversy over whether the haploid number of chromosomes contains a single α chain gene or duplicated α chain genes (Lehmann and Carrell, *Br. Med. J.*, **4**, 748; 1968; Koler and Rigas, *Ann. hum. Genet.*, **25**, 95; 1961; Hollan *et al., Nature*, **235**, 47, 1972; Abramson *et al., Science*, **169**, 194; 1970; Milner *et al., Lancet*, i, 729; 1971). The two hypotheses make very different predictions about the genetic basis of the four classical α-thalassaemia syndromes described in the Asian populations among whom α-thalassaemia is most common. These syndromes include an asymptomatic silent carrier state (α-thalassaemia$_2$ trait), a mild hypochromic anaemia (α-thalassaemia$_1$ trait), a moderately severe haemolytic anaemia (Hb H disease), and fatal hydrops foetalis; the deficits in α chain synthesis in each syndrome correspond well to the clinical severity of each disorder. The 'one gene' theory postulates the existence of two forms of α-thalassaemia gene: a 'mild' (α^+-thalassaemia) allele, and a 'severe' (α^0-thalassaemia) allele heritable at a single locus. The four syndromes would then result from various combinations of normal, α^+, and α^0 alleles in the diploid state. The two-gene theory postulates that all α-thalassaemia alleles are α^0-thalassaemia alleles. In increasing order of severity, the four syndromes would then result from inheritance of α^0-thalassaemia alleles at one, two, three or all four of the α chain loci available in a diploid cell containing duplicated α-chain genes.

Since all α chain genes are deleted in hydrops foetalis, the α^0-thalassaemia allele can be regarded as a gene deletion regardless of which model, one gene or two gene theory, is correct. But the one-gene model predicts that a patient with Hb H disease (α^+-thal/α^0-thal) would have half the normal number of α chain genes. The two-gene model for Hb H disease (α^0-thal-α^0-thal/α^0-thal-α) predicts that this

patient would have only one quarter the normal number of α-chain genes.

Kan *et al.* have now compared the different rates at which DNA from normal and Hb H disease patients hybridises to fixed amounts of α chain cDNA. This method of kinetic analysis depends on the fact that formation of double-stranded hybrids between a fixed-amount cDNA base sequence and a homologous base sequence in cellular DNA will depend on the concentration of the cellular DNA sequence (in this case, an α-chain gene) and time. By measuring the percentage of the cDNA input bound into hybrids at various times, one can construct a 'C_0t' curve (C_0t=initial concentration of cellular α chain DNA sequences $\times$ time of reaction); plots of percentage hybridisation against the C_0t values at each time point permit comparison of the relative rates at which cellular DNA from each type of patient hybridises to the α chain cDNA. The $C_0t_{1/2}$ (C_0t value at which 50% hybridisation occurs) of each curve then serves as a measure of the α chain gene content of one DNA sample relative to the others. When these investigators compared normal DNA to Hb H disease DNA, the $C_0t_{1/2}$ for the Hb H sample was roughly four times as high as that for the normal sample implying that the Hb H sample had only one quarter the normal complement of α chain genes.

These data seem to provide direct biochemical evidence supporting the two-gene theory for α-thalassaemia and the existence of duplicated α chain alleles in normal man. But the use of C_0t curve analysis is fraught with technical difficulties when applied to the analysis of genes present in one or only a small number of copies in human DNA. The α chain genes account for only 10^{-7}–10^{-8} of the total base sequences present in the human genome. Hybridisation between α-chain cDNA and these α chain genes thus occurs very slowly, necessitating the plotting of the C_0t values on a logarithmic scale. Two- to four-fold differences are relatively difficult to measure from the plotted curves. Kan *et al.* devised a set of experiments to determine whether their method could accurately

define this range of differences in $C_o t_{1/2}$. By mixing hydrops foetalis DNA (no α chain genes) with normal DNA in known proportions, they created artificial mixtures having a quarter and half the normal concentration of α chain sequences. The quarter mixture gave $C_o t_{1/2}$ values with α chain cDNA identical to those obtained with Hb H disease DNA. These results support the technical validity of the $C_o t_{1/2}$ values measured for the Hb H DNA samples.

The application of the same approach to β^0-thalassaemia would seem to be straightforward. Unfortunately, the δ chain of Hb A_2 ($\alpha_2\delta_2$) is structurally very similar to the β chain (only 10/146 amino acid differences). Forget *et al.* (*Nature*, **247**, 379; 1974) have shown that β chain cDNA cross-hybridises with δ chain mRNA, suggesting close homology between the mRNAs coding for δ and β chains. If the β chain genes were deleted in homozygous β^0-thalassaemia, DNA from such a patient would have half the normal number of 'β-like' DNA sequences ($\beta+\delta$ genes) since each gene appears (from family studies) to be present as only a single copy. This difference, as shown by Kan *et al.* for their DNA mixtures, comes close to the limits of reproducibility. Homozygous $(\delta\beta)^0$-thalassaemia has been described but this condition is exceedingly rare

and DNA from these patients has not yet been available for analysis. Ottolenghi *et al.* (*Proc. natn. Acad. Sci. U S.A.*, **72**, 2294, 1975) have analysed DNA from a patient doubly heterozygous for β^0-thalassaemia and $(\delta\beta)^0$-thalassaemia in whom none, one, two, or three of the four relevant loci could be deleted, depending on whether either or both types of thalassaemia involved gene deletion. Their results showed a definite difference between this patient's DNA and normal DNA but did not clearly distinguish among the various possibilities.

Further clarification of the genetic composition of patients with β^0-thalassaemia will probably occur only when DNA from individuals with homozygous $(\delta\beta)^0$-thalassaemia can be obtained, or when the content of human globin sequences can be amplified by integration of human globin DNA into bacterial plasmids. The recent progress achieved with α^0-thalassaemia, however, is encouraging. The molecular basis of these disorders appears to be understandable in terms of the number of α chain loci deleted from the genome Perhaps more importantly, the investigation of these syndromes has shown that the same techniques can be applied to resolve issues relating to the number, content, and organisation of globin genes in the normal individual.

actions in free space, on the other hand, must conserve energy and momentum exactly so that they are on the energy shell. A phenomenological nucleon–nucleon potential fitted to the two-nucleon data does not necessarily have the correct off-energy-shell behaviour, and so may not be correct for calculating interactions inside the nucleus when other nucleons are present.

Third, the purely mathematical difficulties involved in calculating the properties of a many-body system from a knowledge of the two-body interaction are so severe that only approximate results can be obtained for any but the simplest system or the simplest nuclear property. It is for this reason that the most important information is obtained from studies of the two bound three-nucleon systems, the helion and the triton. Here it is possible to obtain quite accurate results using a variational calculation or by the methods of Faddeev.

Finally, there is a difficulty in principle that would still remain even if we had a complete knowledge of the nucleon–nucleon interaction and could overcome the difficulties of calculating from it the properties of many-body systems. This is that there may be forces that only come into play in the presence of three or more nucleons. By definition we can never find out anything about these forces by studying the interactions of two nucleons. Indeed, assuming that we can overcome the other difficuties, we can obtain some indication of the importance of three-body forces by seeing how well we can calculate the properties of a three-nucleon system using only two-body forces. If there is a discrepancy, indicating the presence of three-body forces, a three-body force can be included in the interaction and the calculation repeated to see if this gives agreement with the data.

Such three-body forces can be chosen phenomenologically, but here the difficulty is that there are many possible forms, for example $V_3 f(r_{12}+r_{13}+r_{23})$ or $V_3 f(r_{12} r_{13} r_{12})$, where r_{12} is the distance between particles 1 and 2 and so on, and it is not clear what is the best form to take. An alternative approach is to try to calculate the three-body force from meson theory, and use this in calculations of nuclear structure.

In the last few years there have been several sophisticated calculations of the properties of the triton using the best phenomenological potentials fitted to the two-nucleon data, and it has been found that they all give a binding energy that is lower than the experimental value by one or two MeV. Additional calculations show that the binding energy can be varied by as much as 3 MeV by altering the

Nuclear three-body forces

from P. E. Hodgson

ONE of the main aims of nuclear physics is to understand the structure of nuclei in terms of the interactions between the constituent nucleons. The nucleon–nucleon interaction may be studied by measuring the cross sections, polarisations and other characteristics of the scattering of free nucleons, in particular proton–proton and proton–neutron scattering. (It is not practicable to measure neutron–neutron scattering directly since suitable targets cannot be made.) Additional information is obtainable from studies of the only bound two-nucleon system, the deuteron. Very many studies of the nucleon–nucleon interaction have been made over the years in these ways, and the results can be represented by a phenomenological potential that depends on the distance between the two nucleons and on their spins. These potentials cannot be deduced from the experimental results; instead a trial form of the potential is postulated using forms suggested by meson theory and its parameters are adjusted to give the best fit to the data. The potential is thus not unique, and in fact several potentials have been found that give a good overall fit to the data on the

interaction of two nucleons.

The next stage is to use this potential to calculate the properties of nuclei consisting of three or four nucleons, and to see how well the results agree with measurements on the nuclei themselves. There are several difficulties in carrying out this programme. First our knowledge of the nucleon–nucleon interaction is incomplete and subject to ambiguities. This is not too serious since it is possible to compare the results obtained by using different nucleon–nucleon interactions and if they are essentially the same it is reasonable to assume that the differences between the interactions are not important for the nuclear property under consideration.

Second, in a many-body system a nucleon–nucleon interaction takes place in the field of other nucleons, which can absorb momentum. A collision can than occur in which energy is not conserved by an amount ΔE, provided it takes place in a time Δt related to the energy uncertainty by $\Delta E \Delta t < h$. This is expressed by saying that nucleon–nucleon interactions inside the nucleus can take place 'off the energy shell'. Nucleon–nucleon inter-

off-energy-shell behaviour, but if this is done so as to give the correct binding energy the charge distribution no longer agrees with that found from electron elastic scattering. On the other hand if the off-energy-shell behaviour is adjusted to give correct charge distribution the binding energy becomes unacceptably low. It is thus not possible to obtain a simultaneous fit to the binding energy and the charge distribution by varying the off-energy-shell behaviour.

These calculations therefore suggest that possibly the three-body forces contribute significantly to the structure of the triton, and recently Yang (*Phys. Rev.*, **C10**, 2067; 1974) has made a detailed calculation from meson theory to investigate this. He took into account the emission and absorption of pions to lowest order as well as exchange of a ρ meson with a pion and the scattering of a nucleon into an N* excited state at 1,236 MeV. He then evaluated the contribution of the resulting three-body potential to the binding energy of the triton using a variational triton wave function and found that it gives an important contribution of 2.32 MeV to the bonding energy. This is indeed more than enough to bind the triton, the excess being about 0.3 to 0.8 MeV, but it is likely that this is due to higher-order processes not taken into account by Yang.

These calculations suggest that the major part of the discrepancy in the calculated binding energy of the triton can be accounted for by taking three-body forces into account. Furthermore, the contribution of the three-body forces is appreciable, amounting to about a third of the total binding energy. This conclusion is highly unwelcome, since it suggests that the three-body forces, which are complicated and difficult to handle, are likely to be important in a wide range of nuclear structure calculations.

The current work on proton knockout reactions can also give information on the importance of three-body forces. Koltun and others have shown that if only two-body forces operate the total energy of the nucleus may be expressed as a sum of the nucleon removal and kinetic energies over all the occupied orbits. A recent analysis of experimental data for the (e,e'p) reaction on ^{12}C at 497 MeV (*Nature*, **250**, 105; 1975) showed that for protons this sum rule gives −4.0±0 5 MeV for the mean binding energy per proton compared with the accurate value of −6.93 MeV obtained from the nuclear masses with Coulomb corrections. One possible explanation is that there are some fragments of the single-particle strength so far away from the main peak that they escape detection, so that the real

centroid energy is appreciably different from the apparent centroid energy. Another explanation is that three-body forces, which were not taken into account in the derivation of the sum rule, are indeed important. A rough estimate however indicated that the contribution of the three-body forces is small, so it was concluded that the former explanation is the correct one. The results of Yang now make it likely that three-body forces must be taken into account in the derivation of the sum rule.

Another indication of the importance of three-body forces comes from the calculations of Blatt and McKellar (*Phys. Lett.*, **52B**, 10; 1974) They studied the contribution of three-body forces to the binding energy of nuclear matter, and found that it is enhanced if the nucleon–nucleon correlations are taken into account. In this calculation

they used the correlation function derived from the Reid soft core potential, that has been obtained by systematically fitting a large body of data on nucleon–nucleon interactions. Their most surprising result is that the two-pion exchange three-body forces contribute as much as 6 MeV to the binding energy of nuclear matter. This result shows that the Reid potential overbinds nuclear matter when three-body forces are included. Since higher order three-body force terms and four-body force terms are likely to be negligible this indicates that the Reid potential needs to be modified to bring it into accord with all the data.

All these calculations indicate that it is not justified to neglect the contributions of three-body forces, and that they will now have to be taken into account in many nuclear reaction and nuclear structure calculations.

Deterministic models with chaotic dynamics

from Robert M. May

IN nature there are many situations where population growth is a seasonal thing, with no overlap between generations. For instance, many temperate zone insects produce one short-lived adult generation each year, and the population trajectory consists of a sequence of discrete points. Periodical cicadas, with 7, 13 or 17 year intervals between generations, are an extreme example. In mathematical models for such systems, time is a discrete rather than a continuous variable, and one has difference equations rather than differential equations: the population at generation $t+1$, N_{t+1}, is related to that at t, N_t, by some equation of the form

$$N_{t+1} = F(N_t) \qquad (1)$$

Li and Yorke (*Am. Math. Monthly*, in the press) have recently proved a remarkable theorem about any such difference equation. If there is a three-point cycle (that is, a solution such that $N_{t+3} = N_t \neq N_{t+1} \neq N_{t+2}$), it necessarily follows that for the same parameter values there are cycles of every integer period, and furthermore there exist an uncountable number of initial points for which the system is not even asymptotically periodic! Li and Yorke aptly entitle their paper "Period Three Implies Chaos".

Specifically, consider the equation

$$N_{t+1} = \lambda N_t \exp (- a N_t) \qquad (2)$$

This describes a population which at low densities grows by the multiplicative

factor λ ($\lambda > 1$), but at high densities has a propensity to population decrease (the exponential form being plausible if epidemics tend to sweep the population at high densities). This equation has arisen as a model for fish (Ricker, *J. Fish. Res. Bd. Can.*, **11**, 559–623; 1954), periodical cicadas (Lloyd et al., unpublished), and insect populations in general (Moran, *Biometrics*, **6**, 250–258; 1950; Cook, *Nature*, **207**, 316; 1965). The full complexity of its dynamical behaviour has, however, only recently been laid bare (May, *Science*, **186**, 645–647; 1974). For $7.34 > \lambda > 0$, the population is stable about a constant equilibrium value, to which it will tend to return if perturbed. Beyond $\lambda = 7.34$, this stable point bifurcates, and the population alternates up and down in a stable two-point cycle; this two-point cycle in turn becomes unstable, giving way to a stable four-point cycle, then an eight-point cycle, and so on; eventually the range $14.77 > \lambda > 7.34$ is filled by a hierarchy of stable cycles of periods 2^n. For $\lambda > \lambda_c = 14.77$ a chaotic regime is entered: for any given value of λ there is an infinite number of different periodic trajectories, along with an uncountable number of initial points for which the system never settles into any cycle. Further into this chaotic region, for $\lambda > 22.25$, there is a three-point cycle and consequently every integer period is represented.

At this point, proud owners of sophisticated pocket calculators may care to pause and verify some of these statements.

The above phenomena are not patho-

logical consequences of the particular equation (2), but are generic properties of any difference equation (1) describing a population with a propensity to grow at low densities, and to crash at high densities. Among many other examples which could be culled from the entomological literature is

$$N_{t+1} = a\,N_t - b\,N_t^2 \qquad (3)$$

This could be regarded as the simplest nonlinear difference equation (see, for example, Chaundy and Phillips, *Q. J. Math.*, **7**, 74–80; 1936). It has a spectrum of dynamical behaviour similar to equation (2), namely a stable point if $3 > a > 1$, stable cycles of period 2^n if $3.57 > a > 3$, chaos for $4 > a > 3.57$ (and here N tends to $-\infty$ for $a > 4$).

Practical questions aside, these equations have intrinsic mathematical interest in providing simple examples of multiple bifurcation processes. Other examples are much more recondite and intractable.

Another way of describing the chaotic regime is to observe that arbitrarily close initial population values can ·lead to population trajectories which, as time goes on, diverge widely. Even if we have a model which is very simple and fully deterministic, with all parameters assumed to be constant and exactly known, the future is unpredictable. As emphasised by Oster, the dynamics of the system are in many respects indistinguishable from the sample function of a random process, and are best described in stochastic terms.

At present, apparently random fluctuations in animal populations are attributed to environmental vagaries, or to sampling errors. It may be that in the real world the nonlinearities in population growth equations are not sufficiently severe to carry them into the chaotic regime, but the possibility at least deserves attention. Other areas in which similar difference equations have recently arisen are: epidemiology (Hoppensteadt, *CBMS Regional Conf. Ser. appl. Math.*, **20**, 1975); demography (for example, Lee, *Demography*, **11**, 563–585; 1974); macroeconomics (for example, in nonlinear generalisations of the Harrod–Domar model); genetics (Rocklin and Oster, *Am. Nat.*, in the press). In all these examples, the possible occurrence of chaotic solutions needs to be considered.

Chaotic dynamics arising from bifurcations in deterministic models have been noted in other situations. Thus Lorenz (*Tellus*, **16**, 1–11; 1964) has discussed a set of three coupled nonlinear differential equations as a very much oversimplified model of convection processes in the atmosphere, and has drawn from the ensuing chaotic ·solutions the moral that long-term weather forecasting may be infeasible even if one had a simple deterministic model. The details of the bifurcation process here are much more complicated than in simple equations such

as those above, and are still being unravelled (for example, McLaughlin and Martin, *Phys. Rev. Lett.*, **33**, 1189–1192; 1974). Likewise Ruelle and Takens (*Comm. Math. Phys.*, **20**, 167–192; 1971) have proposed a set of four deterministic coupled nonlinear differential equations, with a complex bifurcation structure, as a paradigm for the transition from laminar to turbulent fluid flow. And in an ecological context, Hassell and Comins (*Theor. Pop. Biol.*, in the press) have followed up the above work by exploring the way chaotic population dynamics arise somewhat more easily in discrete time models of two competitors, and Beddington, Free and Lawton (*Nature*, **255**, 58–60; 1975) have done a similar job for discrete time predator–prey models.

Apart from implications in specific scientific contexts, there are general morals to be drawn from the rich spectrum of dynamical behaviour of simple equations such as (2) and (3). Most students come away from elementary mathematics and physics courses (which emphasise linear models) with the impression that simple deterministic models have simple deterministic dynamics. This is, in my view, a very misguided prejudice to bring to biology, economics or sociology, where the simplest models are likely to be nonlinear, and consequently can have strange dynamics. Equation (3) could be studied by high school students before they met differential calculus: it would do them good.

I conclude with some cocktail party chit-chat. Note that the properties of equations such as (2) and (3) in the chaotic regime help to reconcile the Calvinist view of a foreordained, deterministic Universe with the appearance of free will in the world: the Calvinist God knows the exact initial conditions. Less flippantly, one may recall the objections to the probabilistic foundations of modern quantum mechanics, summarised by Einstein's objection: "God does not play dice". Many people have speculated that there may be 'hidden variables', and an underlying deterministic structure. Equations such as (2) and (3) can provide very simple examples of fully deterministic systems whose dynamics are best described probabilistically, and may go some little way toward adding substance to these speculations.

New confidence in ancient pollen data

from Peter D. Moore

THE pious hope of all who study past vegetation by the use of pollen analysis is that the abundance of the pollen of a particular taxon within a deposit bears a close relationship to the contemporary abundance of that taxon in the

vicinity of the deposit Yet, despite the palaeoecologist's declared belief that the present is the key to the past, the study of the relationship between modern pollen rain and the vegetation responsible for it has been sadly neglected. As a result, most of the vegetational interpretations appended to accounts of pollen stratigraphy are largely based upon glib and unjustified assumption.

But information on current pollen rain is now being collected, and many palynologists must have breathed sighs of relief when they saw data such as that published by Lichti-Federovich and Ritchie (*Rev. Palaeobot. Palynol.*, **7**, 297; 1968). These showed that surface sediment samples taken from the major vegetational zones of northern North America contained pollen which broadly corresponded in composition to the vegetation of those zones. Treeless tundra sites proved, predictably, an exception since long-distance transport, particularly of gymnosperm pollen, was a major component of the pollen rain.

Since that study, information on pollen rain has been collected elsewhere from a variety of vegetation types. Webb, for example (*Ecology*, **55**, 17;

1974) sampled the surface sediments of 64 lakes, evenly distributed over a study area in lower Michigan. He could draw fairly precise isofrequency contours of the major tree pollen types across lower Michigan and these corresponded reasonably well with relative abundance maps of the trees concerned. The exceptions were pine and poplar, which were respectively over- and under-represented in the surface sediments. The general north–south gradient in vegetation and in pollen spectra was confirmed by principal components analysis.

Information of this sort causes British palynologists to be filled with a new confidence in the usefulness of that painstakingly accumulated assemblage of fossil pollen data (now safely stored in the Quatenáry data bank at Cambridge). As a result some of these data have recently been dusted down and subjected to fresh scrutiny and analysis by Birks, Deacon and Peglar (*Proc. R. Soc. Lond,.* **B189**, 87; 1975). Basically they have attempted to reconstruct pollen rain maps of the British Isles of 5,000 years ago, using isofrequency contours based on the relative proportions of tree pollen in contemporary peats and lake sediments.

The choice of the period for study, around 3,000 b.c., is a consequence of the recent collation by Smith and Pilcher (*New Phytol.* **72**, 903; 1973) of radio-carbon dates from the major pollen stratigraphic horizons in Britain, which have been used as the basis for the Godwin zonation system. As expected, their collation served to demonstrate the metachroneity of these boundaries in different parts of the British Isles, with the notable exception of the elm decline horizon. This horizon (formerly used to demarcate Godwin's zone *VIIa* from zone *VIIb*— the 'Atlantic-Sub-Boreal transition') was generally dated at between 5,400 and 5,100 b.p. throughout most of the British Isles. It therefore provides a very useful datum horizon in pollen stratigraphic profiles, which is why Birks *et al.* have chosen the levels preceding the elm decline for their analysis.

One feature to emerge from their maps is the paucity of sites from which pollen data are available in the midlands and the south of England compared with the north, the west, and Ireland. This lack of sites means that the placing of isopollen contours in the midlands and south has to be somewhat arbitrary.

The overall picture conveyed by the maps is one of a vegetational gradient within the British Isles in which birch and pine are concentrated in northern and eastern Scotland (with an interesting 'island' of pine in East Anglia),

oak in northern England, elm and hazel in Ireland and the south-west, and lime in south-east Britain. This north-west–south-east gradient is confirmed by principal components analysis of the data; 54% of the total variance in the data is accounted for by the first three principal components, which reflect the major vegetational regions of the British Isles when plotted on a map. The remaining variance is presumably due to local site factors which produce heterogeneity within the regional gradient.

Although there is a greater abundance of data since Godwin (*New Phytol.*, **39**, 370; 1940) first performed this type of mapping exercise, the conclusions are very similar. What has changed is the confidence with which one can interpret these pollen maps of the period prior to the advent of Neolithic man.

Eukaryote transcription initiation sites

from Harry R. Matthews

THE control of gene expression in higher organisms remains a mystery in molecular terms. One of the stages at which control can be exercised is transcription of the gene (DNA sequence) into an RNA sequence which can then be processed and eventually translated. The cell controls transcription in two ways: it avoids transcribing DNA which has a function not expressed through transcription; and it concentrates transcription on those genes that are active or that are controlled at a later stage in expression

DNA is transcribed by an enzyme, DNA-dependent RNA polymerase. Higher organisms possess several such enzymes which are usually classified, operationally, on the basis of resistance to the drug, α-amanitin. Class A, or I, enzymes are resistant; class B, or II, enzymes are very sensitive; and class C enzymes are fairly sensitive. Class A enzymes transcribe the genes for ribosomal RNA and are located in the nucleolus; class B enzymes probably transcribe the bulk of the remaining expressed DNA; and class C enzymes may transcribe the reiterated genes for tRNA and 5S RNA. How does RNA polymerase B know which DNA sequences to transcribe? It seems unlikely that the enzyme itself is responsible for the entire range of tissue-specific transcription that can be observed by molecular hybridisation techniques since, for example, some tissue-specific transcription can be observed *in vitro* using chromatin from different mammalian tissues and bacterial RNA

polymerase. However, especially in the light of reports that a large proportion of DNA in chromatin is available for protein interactions, RNA polymerase B could well need a built-in specificity for particular initiation sites on DNA in order to limit the amount of DNA that needs to be covered by the tissue-specific control elements.

Evidence for such specificity is accumulating. Meilhac and Chambon (*Eur. J. Biochem.*, **35**, 454; 1973) and, now, Cedar (*J. molec. Biol.*, **95**, 257; 1975) have shown that there is a limited number of initiation sites for RNA polymerase B on homologous calf thymus template. Meilhac and Chambon allowed initiation to occur by incubating DNA and polymerase in the absence of nucleoside triphosphates, then adding the rifamycin derivative AF/013 which prevented further initiation and nucleoside triphosphates which permitted chain elongation. The number of RNA chains synthesised was determined from the incorporation of γ-^{32}P-purine triphosphates. They found, on average, one initiation site per 60.000 nucleotide pairs. Cedar has confirmed this result by using high salt conditions to prevent re-initiation of RNA chains after a pre-incubation in low salt, a method first used by Hyman and Davidson in a prokaryote system. He found one initiation per 30.000 nucleotide pairs. Cedar also counted his RNA chains by measuring ^{3}H-UTP incorporation and dividing by the RNA molecular weight obtained by sucrose gradient ultracentrifugation. This method gave one initiation per 40,000 nucleotide pairs.

The figures are acceptably close and correspond to 150.000 polymerase B initiation sites per diploid calf nucleus. The upper limit, given by population genetics, for the number of independent genes per haploid genome is 50,000, that is, 100,000 per diploid nucleus. The number of sites is intriguingly close to the number of genes but, wisely, nobody is jumping to conclusions at this stage. In a long nucleotide sequence a given sequence of seven nucleotide pairs has, to a first approximation, a probability 1 in 65,000 of occurring by chance. Cedar found one initiation site per 50,000 nucleotide pairs with calf thymus B and *E. coli* DNA so the polymerase may recognise a nucleotide sequence about seven nucleotides long that occurs by chance in *E. coli*.

The number of initiation sites is further restricted in chromatin. On average, one initiation was found for every 400.000 nucleotide pairs in calf thymus chromatin in solution, which implies 15,000 initiation sites per diploid nucleus in calf thymus. This is comparable to the estimated number of active genes although it may be a little higher. The

number of initiation sites could be higher than the number of genes expressed if the cell transcribes sequences that do not contain a gene or if many genes have multiple initiation sites. It is also possible that some or all of the initiations observed *in vitro* do not occur *in vivo*. In order to investigate the relationship between the *in vivo* and *in vitro* RNA transcripts their nucleotide sequences must be studied. It is becoming possible to do this directly and some 5'-terminal sequences of nuclear RNAs are appearing (see *Nature*, **255**, 9; 1975). Alternatively molecular hybridisation techniques can be used, especially when a particular gene can be isolated or a cDNA copy of a pure messenger RNA obtained, which can then be used as a highly specific probe for the transcription of a particular gene.

Breeding for protein yield and quality

from Donald Boulter

THE relative importance of protein, as opposed to protein/calorie deficiency, on a world scale, continues to be debated (P. Payne, *New Scient.*, 7 November, 1974), although most of the arguments are of the paper and pencil calculation type. Whatever the final outcome of this debate, it should not obscure the fact that most field workers agree that there is a need to increase the productivity and to stabilise the yield of high protein crops, particularly of legumes since these can fix atmospheric nitrogen.

In diets in many developing countries, legumes play a significant part in supplying protein, as well as in improving soil fertility, and the first priority is to stabilise yield by breeding more resistant lines and then to increase productivity by improved management and restructuring of the plant type. A likely result of such improved legume varieties is an increase in protein yield. Where small amounts of food are required, for example by young children, the total amount of protein consumed is small and any nutritional deficiency of that protein prevents complete utilisation of it. In this situation an improvement in protein quality is essential; this applies both to cereal- and legume-based diets. With adults the available information on diets is insufficient to show the extent of the need for improved protein quality. Also, alternatives such as influencing dietary patterns so that protein from one source may nutritionally supplement that from another, will also have a role. However, in diets in which the main staple is cassava and possibly in those using other roots or

tubers, the quality of the protein consumed may be inadequate for its complete utilisation; here sulpho-amino acids are likely to be first-limiting.

Many of the problems involved have been discussed recently at the Third Research Coordination Meeting of the FAO/IAEA/GSF Seed Protein Improvement Programme, held in Hahnenklee, FDR, 5–9 May 1975

The plant breeders at the meeting were firm in their belief that, to be acceptable to farmers, varieties improved with respect to protein quantity and quality must have high and dependable yields. The one exception to this generalisation is where such varieties might be grown as premium crops where quality brings additional income, a relatively uncommon situation particularly in developing countries. The long-assumed negative relationship between protein yield and grain yield is now known not to be generally valid and in wheat and rice, for example, improvement in both characters has been achieved simultaneously. As long as the demand in a cereal crop for protein content is not excessive, a few percentage points increase, significant advances can be made without sacrificing grain yield. In soyabean, restructuring has achieved very high protein content and high yields.

The same cannot be said, at this time, for protein quality. The experience with high lysine maize, barley and sorghum all point to somewhat reduced yields of grain when genes for increase in lysine are included in a genotype. But it was generally agreed that attempts to incorporate high lysine genes into other genetic backgrounds were only beginning.

J. Axtell (Purdue University) described investigations on natural and induced genes for high lysine in grain sorghum. In this work, a population of seeds was treated with the mutagen diethyl sulphate and selections were made in later generations for the opaque endosperm character by passing seeds over a light box Only certain opaque endosperm genotypes also carried the high lysine character. The desirable endosperm types have been grown for additional generations, but some showed highly deleterious characters as well. Nevertheless, a high lysine endosperm type with good growth characteristics has been recovered (P–721).

R. A. Olson (University of Nebraska) had documented considerable variability in soil conditions in fields used for growing breeding materials for protein improvement, and showed that, probably as a result, the protein content of the plants varied. In the same vein, W. Gottschalk (University of Bonn) presented results from field pea

genotypes in which ranking in both protein content and grain yield were shown to fluctuate widely from year to year. This result is in contrast to those obtained with wheat and barley trials carried on elsewhere, wherein the rankings remained relatively constant between locations and years. Walther (Grünbach, FDR) presented results with barley showing that in an experiment with adequate replication in seasons and locations, it is possible to elucidate plant responses with respect to yield and protein content with apparent reliability. All of these results taken together re-emphasised the difficulties in protein breeding due to gene/ environment interactions, and the necessity for interpreting early results cautiously.

L. Munck (Carlsberg Laboratories, Copenhagen) considered the prospects for the use of high-lysine grains in the animal feeding industry. First, the industry must appreciate the nutritive advantage of high-lysine grains when considering the inventory of the available feeding materials. Second, evaluating procedures need to be available; for example, Munck suggested dyebinding, used in parallel with Kjeldahl nitrogen determination, as a possible means of recognising high-lysine feedstuffs. Only then would feedback pressure on the plant breeders lead to the general inclusion of the high-lysine character in their breeding objectives.

It is well known that legumes are nutritionally deficient in sulpho-amino acids, and D. Boulter (University of Durham) pointed out that it is necessary to consider both sulpho-amino acids, methionine and cysteine, in work on breeding for improved protein nutritive value in legumes. It seems possible to use total seed sulphur as a coarse ranking indicator of the sulpho-amino acids for screening of some legumes In those legumes which contain relatively large amounts of S-methyl-cysteine, sulphur must be determined after removal of this amino acid by extraction with 70% alcohol. Both Boulter and R. M. Gillespie (CSIRO, Glen Osmond, South Australia) suggested that there are reasonable prospects for being able to breed for improved nutritional value by altering the proportions of the major seed proteins which contain different amounts of sulpho-amino acids. In Boulter's view however, present indications are that the range of variation in the protein sulpho-amino acid content of legumes in breeding programmes is small enough to warrant considerable effort in the assembling of world collections.

A new dimension to this problem was furnished by the work reported by Gillespie (see Blagrove and Gillespie, *Aust. J. Plant Physiol.*, **2**, 13–27; 1975;

Gillespie and Blagrove, *Aust. J. Plant Physiol.*, **2**, 29–39; 1975). They have found that in lupins the relative proportions of the major seed proteins can be changed up to a point by supplying sulphur at different levels; in high-sulphur treatments the proportion of a high-sulphur protein was increased. Thus, there is a strong gene–environmental interaction, although whether the effect is directly on gene expression at the transcription or translation levels, or whether it operates by a changed physiology, is not clear. It will be of great interest to see how general these results are for other legumes.

T. Behre (Debre Zeit, Ethiopia) reported a breakthrough on breeding *Eragrostis tef*, which represents a significant source of protein in Ethiopia. Up to now, all attempts at controlling the pollination of this crop have been unsuccessful. But as a result of studying the basic reproductive process of the plant, it has been possible to perform hand pollinations, so that normal procedures of plant breeding may be applied and it should now be possible to make improvements in yield through breeding.

In the realm of improved techniques for screening and evaluating the nutritional quality of advanced lines, there was an encouraging report concerning the use of a fluorescence technique for the specific determination of lysine in ground samples of grain. Less advanced is some work on a similar technique for tryptophan determination. Both of these developments were reported by A. K. Kaul (Institut fur Strahlenbotanik, Hannover).

A number of contractors from developing countries reported progress on the development of improved protein genotypes in several crops, including bread and durum wheat, rice, barley and several legumes. Because of the early stage of the work, no attempt is made to detail the results here, but a general feeling of optimism prevailed For example, H K. Jain (New Delhi) was convinced that tropical legumes as a group were excellent materials in which great improvements could be achieved.

A homogeneous Universe?

from Malcolm MacCallum

THE simplest cosmological models assume the Universe to be spatially homogeneous and isotropic Isotropy, the equivalence of all directions from us, is on a fairly firm experimental basis provided by observations of the cosmic microwave background, the X-ray background and so on, although there are contradictory results (Brown, *Mon.*

Not. R. astr. Soc., **138**, 527; 1968; Wilson, *Mon. Not. R. astr. Soc.* **155**, 275, 1972) demanding further investigation. However, isotropy does not directly imply homogeneity, the equivalence of all points in space, for we might simply be at 'the centre of the Universe'. There is a strong anti-geocentric prejudice which favours homogeneity, but a lack of observational evidence.

The prejudice itself is recent. Only in 1918 did Shapley find the Sun was not at the centre of our Galaxy, three and a half centuries after Copernicus's discoveries. It was 1924 when Hubble proved some 'nebulae' were galaxies and 1952 when Baade's revision of the cosmic distance scale showed other galaxies could be as large as our own. The 'Copernican principle' and its strong form, the assumption of homogeneity, have subsequently been almost articles of faith, especially as they have the practical advantage of simplicity.

This attitude is reinforced by the difficulties of observationally checking homogeneity, arising because it involves a knowledge of conditions at all points, however distant. All one can hope to say is that any inhomogeneity has a scale greater than our range of observation. If the latter included our whole past we would know all we really require. But the finite velocity of light means we must compare distant objects as they were in the past and not as they are now, so we need to know about the evolution of both individual astrophysical objects and the Universe as a whole to make the comparison

In spite of these difficulties, our view about homogeneity is naturally influenced by knowledge of the cosmic structure in our vicinity. The most obvious feature to test is the distribution of galaxies. It is now well known that galaxies are clustered and so not homogeneously distributed. de Vaucouleurs (for example in *Science*, **167**, 1203; 1970) has expounded a theory of a hierarchy in which clusters are grouped in superclusters and so on Peebles and collaborators (*Astrophys. J.*, **196**, 1; 1975) found no evidence of a hierarchy in their extensive statistical investigations of catalogues of galaxies. They concluded that there is a continuous spectrum of clusters on scales $10/h$ kpc to $5/h$ Mpc, where h represents the time cosmic distance scale in suitable units and 1 pc is roughly 3×10^{13} km. Current estimates of h are roughly in the range $\frac{1}{2}$ to 1. This distribution could be the effect of gravitational attraction within initial accidental density enhancements, the clusters themselves being homogeneously distributed.

Turner and Gott (*Astrophys. J.*, **197**, L89; 1975) have just announced a new development. They were studying the problem of the non-luminous 'missing mass' that may be required to gravi-

tationally bind clusters of galaxies, by examining the dynamics of small groups of galaxies. To find such groups they took all Northern Hemisphere galaxies brighter than fourteen magnitude (which corresponds to a distance of order roughly 100 Mpc) at more than forty degrees from the galactic plane, and separated these into two classes according to whether their nearest neighbour was at an angular separation greater or less than 45 minutes of arc They noted that the more isolated class appeared to be homogeneously distributed in the sky, and thus tried applying Peebles's statistical methods to each class separately. They found the 'isolated' class, about 40% of the total, were indeed homogeneous, once effects of galactic obscuration and statistical noise had been allowed for. The other class however showed strong clustering on scales up to eight degrees.

This result, which invites further examination, will, if confirmed, leave us awkwardly poised. Each of the two classes separately is reasonably explicable, but Turner and Gott show from velocity and brightness data that they must be intermingled, and how one class became clumped while the other did not is a mystery. Once again nature proves to be more complex than was thought.

Of bedsteads and neutrons

from James Binney and Philip Candelas

INTEREST in the interaction of quantum theory and gravitation has been growing recently. Following Hawking's sensational prediction (*Nature*, **248**, 30; 1974) that black holes may emit photons and other elementary particles, several recent publications have discussed the importance of quantum effects in strong gravitational fields (see review by DeWitt, *Physics Reports*, in the press; and *Quantum Gravity: An Oxford Symposium*, edit. by Isham, Penrose, and Sciama; Oxford University, 1975).

Just to make sure we have the basics right Colella, Overhauser and Werner (*Phys. Rev. Lett.*, **34**, 1472; 1975) have been checking up experimentally that the de Broglie waves associated with neutrons really are refracted by a gravitational field in the same way that proton wavepackets are refracted by an electric field. Colella and co-workers have cut from a single crystal of silicon a device which operates much as a Young's double slit experiment. Bragg diffraction in one part of the crystal splits a neutron beam into two, each being subsequently diffracted back towards the other by another part of the crystal. Colella *et al.* then monitor

the intensity of the signal produced by the interference of these two beams in a third part of the crystal. They find that the relative phase of the two interfering beams changes as elementary quantum mechanics predicts it should when the apparatus is rotated so as to alter the relative inclination of the two beams to the horizontal.

Of course one is not very surprised that the result of a low-energy experiment of this type is accurately predicted by conventional quantum theory. It is unfortunate that weak gravitational fields should be the only ones accessible to experiment since recent remarkable results due to Hawking and Beckenstein indicate profound interrelations between quantum theory, gravitation and thermodynamics.

Hawking discovered that, in the case of a realistic black hole formed by stellar collapse, effects to do with the quantum fluctuations of the vacuum cause real particles to be produced and emitted to infinity as a constant flux. What is really astonishing about Hawking's result is its form. The outcome of his highly sophisticated analysis is that black holes should radiate photons and other particles precisely as if they were black bodies of definite temperature. The temperature is inversely proportional to the mass of the hole.

Surprisingly, this result is essentially that suggested earlier by Beckenstein (*Phys Rev.*, **D7**, 2333; 1973) from an entirely different, very classical argument : Take a large, brassy Victorian bedstead and consider it carefully for a moment. You observe four legs, many knobs and springs. Indeed if you are a connoisseur of such things you might well guess that it was manufactured in Bristol in 1864. Toss this same bedstead into a black hole and all this information is suddenly lost to you. The only differences that you observe in the hole are small changes in its mass and angular momentum. But of course a loss of information implies an increase in entropy, an effect which one can quantify. In this way Beckenstein was able to put a value on the entropy of a black hole, which turned out to be proportional to its surface area. From this followed in a formal way a value for its thermodynamic temperature. What Hawking showed us was that this formal temperature is perfectly real.

Thes two results fit beautifully with work by Bardeen, Carter and Hawking (see for example Carter's contribution to *Black Holes*, edit. by DeWitt and DeWitt; Gordon and Breach, 1973) who showed that changes in the configuration of a black hole are constrained by a series of laws analogous to those of thermodynamics. This similarity was at first thought entirely

formal with the inverse of the black hole mass having the role of temperature and the area of the black hole behaving like entropy. Now in the light of the work by Hawking and Beckenstein we see that there is a complete identification of black hole parameters and thermodynamic variables.

It is very encouraging that a field as young as is the study of quantised particles in gravitational fields should already have produced a much needed piece of the jig-saw of physics. A host of cosmological problems seem to require for their solution a better understanding of these quantum processes. Perhaps with the solution of these other problems we will again obtain important and unexpected insights into the nature of things.

Spots before the eyes

from E. G. Richards

A RECENTLY published two-dimensional electropherogram shows the resolution of more than 1,100 proteins from *E. coli*. This spectacular result is to be found in a paper by O'Farrell (*J. biol. Chem.*, **250**, 4007; 1975):

T w o - dimensional electrophoresis involves the separation of a mixture along the first dimension according to some molecular property of the components; this is followed by movement of the components in a perpendicular direction resulting in separation according to another property. Most attempts to apply such methods to mixtures of proteins have suffered from correlations between the two chosen properties resulting in a tendency of the components to cluster round a diagonal in the final pattern.

O'Farrell has played the simple trick of separating according to isoelectric point by isoelectric focusing in the first dimension followed by a separation according to molecular weight by SDS-polyacrylamide electrophoresis in the second The isoelectric point and molecular weight show virtually no correlation.

To summarise his method, the separation in the first dimension is conducted in a 2½% cylindrical polyacrylamide gel containing 9 M urea and ampholines to produce a *p*H gradient between *p*H 5 and 7. For the second dimension a 0.8 mm thick flat bed gel of polyacrylamide with an exponential concentration gradient and containing SDS is used. The sample to be applied is first treated with nucleases to remove RNA and DNA which otherwise produce streaking effects and then the proteins are solubilised and denatured in 9 M urea before application to the first dimension. After the first separation

the isoelectric gel is advantageously equilibrated by shaking in SDS solution before incorporation into the flat bed gel. Although this equilibration leads to some loss of proteins and some impairment of the resolution, it further reduces streaking.

The authors claim that about 70 bands can be resolved in the first isoelectric separation, and up to 100 in the second SDS dimension. Thus a maximum of 7,000 proteins could in principle be resolved though this figure may be reduced somewhat in practice for a variety of technical reasons. Nevertheless the authors have counted more than 1,100 spots obtained from the application of whole *E. coli* extracts. A rough calculation which assumes a chromosome of 4 million base pairs and a thousand of these to each protein gives an upper limit of 4,000 different proteins in *E. coli*.

Two detection systems were utilised : staining with Coomassie blue and autoradiography with the use of ^{14}C labelled proteins. The first method can detect 0.01 *µ*g of protein in a spot; the second, in a twenty day exposure, can detect 1 c.p.m. which could correspond to a protein which existed in but one copy per cell at realistic, albeit high, specific activities. However these detection limits are by no means the whole story since the picture is complicated when the total load of protein is raised to enable minor components to be detected. In the first place the total protein load must be kept low to avoid streaking effects and other distortions of the spot shape; in the second the size of a spot was found to increase when the amount of protein in it increased. This means that minor components located close to abundant proteins would be hidden and remain undetected.

The authors claim that replicate runs are highly reproducible provided the conditions are carefully standardised and ampholines from the same batch are used. The comparison of two electropherograms is facilitated by the characteristic patterns of spots produced

If the author's recipes are not carefully adhered to, certain artefacts may be introduced such as streaking and multiple spots due to charge heterogeneity and solubility problems.

It would thus sem that a new tool of considerable power has arrived and it will be interesting to see what use is made of it O'Farrell himself says that the method as it stands does not work for basic proteins such as ribosomal proteins and histones but one may guess that there are no insuperable problems in adapting it for such mixtures. Maybe the characteristic patterns of spots will also commend themselves to taxonomists.

review article

Computer-aided thought in biomedical research

W. J. Perkins & B. J. Hammond*

With the technology at present available, computers are potentially more than simply mindless calculating machines. They can now be programmed to make intelligent use of the ideas researchers have about the problems they are trying to solve.

The human mind is seldom satisfied, and is certainly never exercising its highest functions when it is doing the work of a calculating machine. What the man of science, whether he is a mathematician or physical enquirer, aims at is to acquire and develop clear ideas of the things he deals with. For this purpose he is willing to enter on long calculations and to be for a season a calculating machine, if he can only at last make his ideas clearer.

J. C. Maxwell (1870)[1].

THE relationship between the man of science and his calculating machine has undergone, particularly in recent years, a rapid evolution but it is the machine and not the man that has changed. For in spite of the almost overwhelming power of a modern computer, the scientist still simply seeks to 'develop clear ideas of the things he deals with'. The calculating power that evaded Maxwell's grasp has since become a common-place means of evaluating data from scientific experiments but it is equally well established that the computer is, at least potentially, more than an outsize calculating machine. Substance is being added to this view, albeit slowly, by research in those areas of computer science gathered somewhat uneasily under the umbrella of artificial intelligence[2], but for the scientific user the more exciting prospects must at present remain as unredeemed promises. It is nevertheless possible to marshal a sufficient range of techniques, both in hardware and software, to provide the researcher with an effective tool for processing ideas as effectively as he may process numerical data.

An illustration of the difference between these two activities is seen in the application of computers to the problem of virus structure. The electron microscope provides pictorial views of individual viruses seen from various angles but, because of the preparative procedures and the fact that the microscope is operating at its limit of resolution, the images obtained are difficult to interpret in terms of three-dimensional structure. The electron micrograph may, however, be scanned automatically and reduced to an array of density data in a computer, thus allowing various lengthy mathematical transforms to be applied in attempts to improve the resolution of the record, or to make symmetry relationships more explicit. It is also possible to reconstruct the three-dimensional shape of a virus from a series of electron micrographs of a given specimen taken at various tilt angles, though at present, radiation damage of the specimen limits the number of exposures that can be made and so restricts the technique to highly symmetrical structures[3]. This data processing approach is designed to help in building a mental picture of virus structure but it makes little use of the tentative ideas that the researcher may be carrying in his head at any time.

*Address: The National Institute for Medical Research, Mill Hill, London NW7 1AA, UK

Ideas about the possible shapes of a virus find a more direct means of expression in the use of physical models, which act both as a focus and an embodiment of the researcher's thinking and can be changed almost at will as shortcomings of particular structures are recognised. A more sophisticated and more flexible extension of the physical model can be obtained if the model is computer-based but, particularly in the early stages of model development, there is a need for more rapid assessment of ideas than that provided by off-line computer facilities. Close interaction between modeller and model is needed if the computer is to aid and not frustrate creative thinking of the user.

Hardware and software

From the technical point of view then, it is necessary to exploit to the full those computer facilities that speed communication, in either direction, between man and machine so that a computer intended as a thinking-aid is characterised more by its range of operator input–output devices than by an impressive size. Such input devices include, in addition to the standard keyboard, simple switches and potentiometers for indicating binary conditions and analogue levels to the machine, graphic tablets for communicating line contours to the computer, and the light-pens and joy-sticks used in conjunction with output displays to signal positional information. Interactive output facilities, in addition to the standard teleprinter or its electronic equivalent, encompass various oscilloscope devices for displaying line drawings in a storage or non-storage mode and television-type displays for presenting continuous tone or coloured picture information to the user.

The provision of suitable hardware is necessary but not

Table 1 Keyboard commands for rotating computer model of adenovirus hexon

a Building instructions

Command	Description
BR/X./Y./Z./	Bring a cell into position
JN/-N/φ./ψ./	Join on to cell N at given angles
SH/--N/-N1-N2/	Show number of cell N. Type coordinates N1 to N2
DE/--M/--N	Delete cells M to N
MST/M/-N	Make a structure numbered M
BRS/-N/X./Y./Z./θ./ψ./	Bring a structure into position given
BN/--N/	Add background of N dots per inch²
RE/	Read in a structure
OU/	Output a structure

b Rotation instructions

Command	Description
AX/N/φ./θ./	Set angles of axes for quadrant N
RTX/θ./	Rotate structure about X by angle θ
RTY/φ./	Rotate structure about Y by angle φ
RTZ/ψ./	Rotate structure about Z by angle ψ

Fig. 1 *a*, Basic model of adenovirus hexon in the orthogonal planes; *b*, various orientations of the adenovirus hexon model; *c*, electron micrograph of adenovirus hexons showing diverse orientations.

written in autonomous blocks. Modular programming provides a suitable software structure that implements the various model manipulations as a set of self-contained blocks to which changes, additions and deletions may readily be made as modelling requirements change with a model's evolution.

Interactive graphics

One basic way in which hardware and software may be coordinated to provide an aid to thought lies in the use of interactive graphics, which can best be illustrated by returning to the problem of virus structure. A modular program has been developed that allows the interactive building of tentative virus structures using spheres as basic building units. The keyboard commands for the control of model building are shown in Table 1 and the three-dimensional nature of the structure is presented to the user as orthogonal projections on a storage display screen. Starting with a blank screen the user can type bring (BR) commands to establish single spherical units at specified coordinates and further units can be attached to existing units by use of a join (JN) command. Each unit is allocated an identification number as it is entered on the screen and the identity of a specified unit can be retrieved at any time using a show (SH) command. Units can also be removed from the structure by means of a delete (DE) command. Another useful building feature is the ability to mark and store a structure using an MST command which subsequently allows the marked structure to be used again, either in its own right or as the subunit of a grander design.

These few commands, with the immediate presentation of their effects on the model, allow effective interaction between user and computer in building plausible models of virus structure; this is illustrated by Fig. 1*a* which shows a model of an adenovirus hexon, built up in this way and displayed in the orthogonal planes. The human contribution to the man–machine interaction is not only his speculative ability but his ability to compare the geometric properties of a model with the fuzzy images provided by the electron microscope. This comparison can be assisted by randomly filling the spherical building units with a suitably dense distribution of dots to indicate depth. In addition, a random distribution of dots at any selected level can be added as a background to the model. By the use of the further keyboard commands given in Table 1, the model can be rotated about all axes to present planar orientations that can be compared directly with the two-dimensional projections observed in electron micrographs of the virus specimens. The diverse patterns observed during rotation give ideas as to how other patterns might be obtained then the model can be refined to obtain a closer match. Various orientations of the adenovirus hexon model may be seen in Fig. 1*b* and related to the diverse projections observed in the electron micrograph of Fig. 1*c*. The distribution of categorised patterns in the model may also be compared with distributions in electron micrographs for different preparative procedures to assess their effect on the patterns obtained.

Three-dimensional displays

Two-dimensional projections of computer-implemented structures are progressively less useful as three-dimensional complexity increases, but there is no difficulty in transforming the data in the computer to produce a stereo display[4]. An example of this approach features molecular models of proteins where the user, viewing a large screen oscilloscope through a suitable mirror system, sees the appropriate network of straight-line bonds as a three-dimensional structure in space. The space relationships can be further clarified by rotating about orthogonal axes using manual potentiometers interfaced to the computer through A–D converters. This presentation is at first dramatic but is no more than a computer-based version of conventional ball and stick models, that is, until interactive facilities are added to the basic facility.

sufficient to establish close interaction between user and computer; effective interaction also lies with effective programming. A difficulty arises in the fact that in the present context the models are by definition tentative, and the nature of any changes that may subsequently be required cannot be clearly foreseen, yet an interactive program should at the outset provide the user with a sufficient range of alternative model manipulations at least to launch the model on its uncharted course of development. Subsequent program changes will almost inevitably be required, either to modify the nature of the model or to provide further manipulation facilities, and the ease with which these may be implemented depends to a large extent on the degree to which the program has been

Consider for example the molecular conformation of complex molecules where the sequences of atomic components are largely known but where the structure still retains sufficiently numerous degrees of freedom to generate an immense number of possible second order configurations. The configurations preferred by nature are those of minimum energy but the determination of this condition for a complex molecule by an unguided search is usually rendered impracticable by the sheer number of candidates to be considered and by the numerous local minima observed[5]. In practice the researcher seeking natural conformations bounds the search according to largely empirical rules, but if a closely interactive approach is adopted, available clues from other sources may be incorporated together with experience, ideas and sometimes intuition. Three-dimensional displays of the molecular structure provide an effective focus for such interaction and control of the conformation of the structure is readily provided by manual potentiometers that specify particular bonding angles and can therefore be used to rotate one part of the structure relative to the remainder.

At any point in the interaction the user may call for energy calculations to be made corresponding with the incremented rotations of a specified bond about two orthogonal axes. This information can be presented in a variety of ways but each uses the mapping of energy values on to a plane defined by axes of the incremented variables. The more sophisticated displays present the user either with constant energy contours or with two-dimensional views of the energy surface constructed above the reference plane. In either case a rapid assessment of the current situation can be made and serves to direct the next step in pursuit of a minimum energy configuration[6].

Conversational mode

The interactive manipulation of complex molecules places its heaviest technical demands upon communication from machine to man but in other situations, communication in the reverse direction is more important. The man-to-machine link is made through a coded instruction chosen from a prearranged list. If the idea of an instruction is, however, interpreted too rigidly, that is, if it is taken to imply an imperative requiring no response other than blind obedience, then the onus for precise expression of complex instruction formats falls entirely on the user and any operator errors will generally cause the computer program to abort. The consequent loss of rapport between man and machine can, however, be restored by upgrading the role of the computer as an instructed device, so that it apparently takes the initiative whenever a decision is required. At such times the computer explicitly invites a decision, checks the validity of the user's reply and then, by prompting, guides the user along the chosen path of action. In this way a conversational mode of control evolves in which the real prerogative of choice remains with the man, while the tedious responsibility for its appropriate expression is largely taken over by the machine.

An illustration of the conversational mode arises with an interactive model that has been formulated to explore ideas about the establishment of neural connections between the retina of the eye and the tectum during an animal's development. It is known that the retinal cells are eventually mapped, by their connections, in a spatially ordered manner on to the tectum and the model therefore allows the implementation of various schemes by which the ordering process may be controlled[7]. Critical assessment of particular schemes is provided by model manipulations that mimic experiments that have been carried out to perturb the developing retinotectal system by various surgical interventions. Whenever the computer is ready for a fresh instruction it types out the query 'OPTION =' and the user may then reply with a simple mnemonic option code selected from a list of some 40 alternatives. One set of codes allows the current state of the model to be examined by calling for the display of tectal mappings corresponding to various points or straight lines drawn on the retina. Other codes control the sizes and rotations of the retina, tectum and tectal grafts
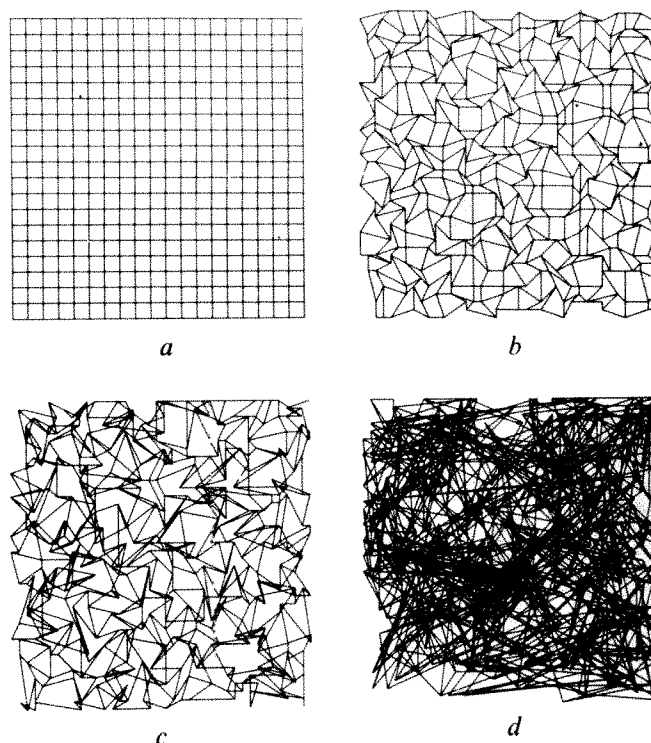
Fig. 2 One of several optional display facilities traces the rows and columns of the current retinal array as mapped on to the tectal array at any time. The result of executing an ordered mapping is shown in *a*, while *b*, *c* and *d* show the display as it appears after each retinal cell termination has been allowed a random walk on the tectum of 1, 5 and 100 steps respectively.

while the initial mapping of retinal cells on to the tectum, in either random or ordered fashion, is controlled by further codes. All option codes are checked on receipt by the computer and in the event of operator errors the user is asked to restate his chosen option; in the absence of code anomalies the computer asks for any further information, usually numerical, necessary to fulfil the particular option. This information is checked before implementation to ensure that impossible conditions, such as mapping the current retina on to too small a tectum, are not implied in the user's choice.

Further, more basic options implement changes in tectal termination positions, for example by allowing each termination the freedom of a random walk. When the random walk option is applied to an initially ordered mapping of retinal cells on to the tectum, the display of a rectangular retinal lattice mapped on to the tectum is subject to ever-increasing randomisation as shown in Fig. 2. This effect accompanies the assumption that, during development, retinal cell terminations possess an innate mobility on the tectal surface and in order to achieve the spatially ordered mapping observed in mature animals, the tectal ordering mechanisms must oppose, indeed reverse, this randomising tendency.

One scheme that has been found to order a random tectal mapping involves comparison of the retinal cell locations corresponding with neighbouring tectal terminations. Each termination is considered in turn and interchanged with a randomly selected neighbour if, and only if, its tectal position with respect to that neighbour contradicts the relative positioning of the corresponding retinal cells. In experimenting with this scheme applied to more complex model conditions some unexpected modes of behaviour have been observed. For example, when half of a tectum is rotated through 180°, so reversing its sense of direction, it was expected that a random mapping of retinal cells on to the compound tectum would
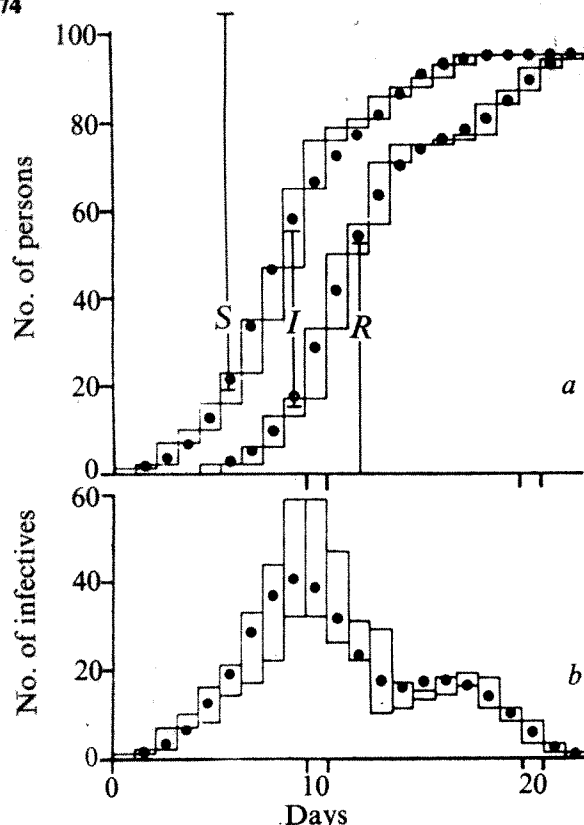
Fig. 3 The rectangles represent the maximum and minimum interpretations of field data recording the daily numbers of persons susceptible to infection (S), the number infected and assumed to be infectious (I) and the number recovered from a common cold infection as functions of time (R). The dots show the corresponding performance of a dynamic model evolved using interactive hybrid simulation.

of simultaneous differential equations. In these cases the addition of an analogue integration capability to an interactive facility can greatly enhance the speed and hence the effectiveness of communication.

The contribution that can be made by analogue integration is illustrated in an example from the field of epidemiology[3]. In spite of a great deal of analytical work on mathematical models of the epidemic process, the validation of even basic model assumptions has been hampered by lack of suitable field data. For such validation, accurate dynamic data are required concerning a small, but not too small, isolated, homogeneous and gregarious community into which a single infection is introduced. This requirement was apparently met when seven sets of data became available concerning common cold outbreaks on the island of Tristan da Cunha. The form of these data is shown in the upper part of Fig. 3. A flexible scheme of differential equations was therefore formulated to embrace some of the alternative ideas used in existing models of the epidemic process and the equations solved by interfacing standard analogue computer modules to perform the necessary integrations. A fast solution time (80 ms) was obtained and warranted the presentation of the solutions on a display screen concurrently with selected sets of field data. This enabled the immediate appreciation of changes in model behaviour corresponding with changes in mechanisms and values incorporated in the model structure.

A model was thus evolved that agreed well'with five sets of field data but the remaining two sets remained enigmatic since experiments with the model showed that the most acceptable explanation lay in the unlikely event of a double infection involving different viruses. Since the most likely source of such viruses seemed to be the occasional visiting ship, the records of ship arrivals were examined and the occurrence of the two anomalous epidemics were found to follow the almost simultaneous presence of two visiting ships. The agreement between model and data in one of the double infection situations is shown in Fig. 3.

eventually be ordered as if half an ordered tectum had been rotated. This idea was promptly revised when the model was observed to behave as two quasi-autonomous tecta that independently ordered their quota of randomly allocated terminations.

Dynamic systems

The eventual explanation of retinotectal mapping must have some dynamic content since changes occur in both temporal and spatial domains. In many other situations the changes that take place with time, eclipse any detailed interest in changes that occur with spatial dimensions and the geometric content of the corresponding models is reduced to the gross representation of structural features as they affect dynamic performance. This transition is highlighted in the use of compartmentation schemes, perhaps most familiar as descriptions of metabolic or drug distribution systems.

The derivation and implementation of dynamic models using computers is a subject of sufficient substance to be recognised as a separate discipline, that of computer simulation. Little of the simulation work on biological systems, however, has been carried out in sufficiently close interaction with the machine to exploit the computer simulation route to computer-aided thought. One reason for this lies in the use of high level computer simulation languages which considerably ease the burden of programming, but in execution the resultant programs are often too slow and inflexible to support on-line thinking. At least at present, custom-built applications programs remain advisable and may conveniently follow the same guidelines, and provide the same interactive facilities, as those required for the effective manipulation of purely geometric models. In terms of hardware, however, the requirements for geometric and dynamic models are not identical since many of the latter, typified by compartmental models, find their natural formulation in terms

Present position

The essence of computer-aided thought is the effective interfacing of the conceptual prowess of man with the manipulative capability of the computer. At present, and possibly for some time to come, the finesse with which this can be accomplished is limited by available technology, but yet provides an effective means for accelerating the development of ideas. The real impediments to this use of computers in biomedical research are thus not basically technological but would seem to be more of human origin. First, the idea of computer-aided thought has not in general been brought to the attention of the biomedical researcher, at least not in a comprehensible way, so that there is little motivation to explore its application to his own work. Second, since specific ideas are to be tested, they require specific programs of some complexity in order to obtain the closeness of interaction needed for this type of application and the average biomedical researcher is rarely willing to devote the time and effort needed to acquire the necessary expertise for this purpose. Last, even where suitable facilities exist, the computer specialist is often distracted by the demands of his own subject from understanding a biomedical problem in sufficient depth to provide the researcher with an appropriate and effective means of interaction with the computer.

These problems are by no means unique, indeed they are quite familiar in other fields including that of bioengineering where it has been recognised that an engineer needs more than a superficial knowledge of biomedical subjects if he is to be maximally effective. Computer scientists specialising in biomedical applications may need to follow a similar pattern if the computer is to be fully exploited as a research tool.

the retino-tectal model, Dr N. G. Wrigley for providing the electron micrograph of Fig. 1, and Dr D. A. J. Tyrell, with whom the epidemiological work was carried out.

[1] Clerk Maxwell, J., *British Association Report*, XL (1870).
[2] Lighthill, Sir James, *Artificial Intelligence* (Science Research Council, London, 1973).
[3] Crowther, R. A., De Rosier, D. J., and Klug, A., *Proc. R. Soc.*, A317, 319-340 (1970).
[4] Perkins, W. J., Piper, E. A., Tattom, F. G., and White, J. G., *Computers and Biomedical Research*, 4, 249-261 (1971).
[5] Katz, L., and Levinthal, C., *A. Rev. Biophys. Bioengng*, 1, 465-504 (1972).
[6] Perkins, W. J., Piper, E. A., and Thornton, J., *Computers in Biology and Medicine* (in the press).
[7] Gaze, R. M., *Br. Med. Bull.*, 30, 116-121 (1974).
[8] Hammond, B. J., and Tyrell, D. A. J., *J. Hyg. Camb.*, 69, 423-433 (1971).

articles

Amplification of a specific region of the polyoma virus genome

Beverly E. Griffin & Mike Fried

Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

Defective DNAs, isolated from mouse cells after infection with a clonally purified stock of defective polyoma virus, have been found to consist of nucleotide sequences from about 17% of the wild-type polyoma genome, tandemly repeated two to six times. These sequences come from the region of the viral DNA which contains the origin of DNA replication and the 5' ends of early and late stable mRNAs.

THE genome of polyoma virus is a supercoiled DNA molecule composed of about 5,200 pairs of nucleotides, and has a coding capacity for approximately 200,000 daltons of

protein. Polyoma virus can undergo at least two different types of interactions with susceptible cells. One interaction results in the death of the cell concomitant with an increase in the number of virus particles (productive or lytic infection). The other results in cells acquiring a set of characteristics which resemble those of tumour cells (nonproductive or transforming infection). When cells that are capable of being lytically infected are treated with high multiplicities of polyoma virus (high virus to cell ratios), virus particles containing non-infectious supercoiled DNA molcules, heterogeneous in size, are produced. With continued high multiplicity passage, particles containing this non-infectious DNA, termed 'defective' DNA, will come to predominate the virus stock[1-3].
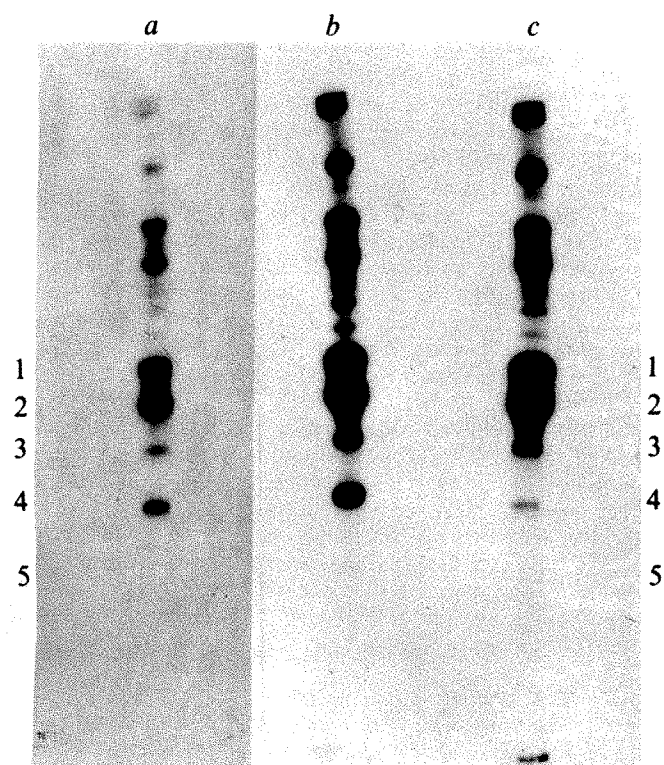
Fig. 1 Autoradiograms showing electrophoretic separation of the different ^{32}P-labelled DNA species obtained following infection of 3T6 mouse cells with D-50 clonal isolates. Virus stock grown from the clonal isolate containing D-50 molecules[3,5] and wild-type helper virus, was used to infect 3T6 cells in low phosphate media in the presence of ^{32}P-phosphate[6]. Viral DNA was selectively extracted from infected cells[15] and supercoiled DNA molecules purified by CsCl equilibrium centrifugation in the presence of EtBr (ref. 16). The band containing supercoiled DNA was isolated and the EtBr removed by extraction with CsCl-saturated 2-propanol[16]. DNA was recovered by alcohol precipitation after diluting the aqueous solution threefold with Tris-EDTA (10 mM-1 mM). This DNA was further separated into a number of species by electrophoresis on 1% agarose cylindrical gels (SeaKem agrose, 0.8 × 15 cm) with E buffer[14]. The various gel bands (1-5) were located by autoradiography, excised, and DNA eluted by electrophoresis into dialysis membranes[17]. The sizes of the DNAs from bands were determined by their sedimentation properties in 5-20% sucrose gradients[3]. By these procedures, the calculated sizes[21] of the various DNA species were estimated to be about 34, 51, 68, 85 and 102% wild type genome length. Bands : 1, 102% defective supercoiled molecule and wild type helper supercoiled DNA; 2, 85% supercoiled defective molecules; 3, 68% supercoiled defective molecules; 4, 51% supercoiled defective molecules; 5, 34% supercoiled defective molecules. (The bands migrating more slowly than Band 1 contain linear and nicked circular forms of the different defective DNAs and various species of mitrochondrial DNA.) Gels: *c*, Supercoiled DNA from 3T6 cells infected with the original clonal isolate[3] of D-50 virus. *a* and *b*, Supercoiled DNA from 3T6 cells infected with a virus derived from purified D-50 molecules and added wild type helper viral DNA. (The D-50 molecules were purified and isolated as supercoiled species first from sucrose gradients[3], followed by CsCl-EtBr density gradient centrifugation[16], before being combined with the wild-type helper DNA to produce the virus stock.) *a* and *b* differ only in the length of time the gel was exposed to the X-ray film.

Columns

1 2 3 4 5 6 7 8

1 (2 7·3)
2 (2 1·4)
3 (1 6·8)
4 (1 3·2)
5 (7·4)
6 (6·7)
7 (5·2)
8 (1·8)

4 %

7 %
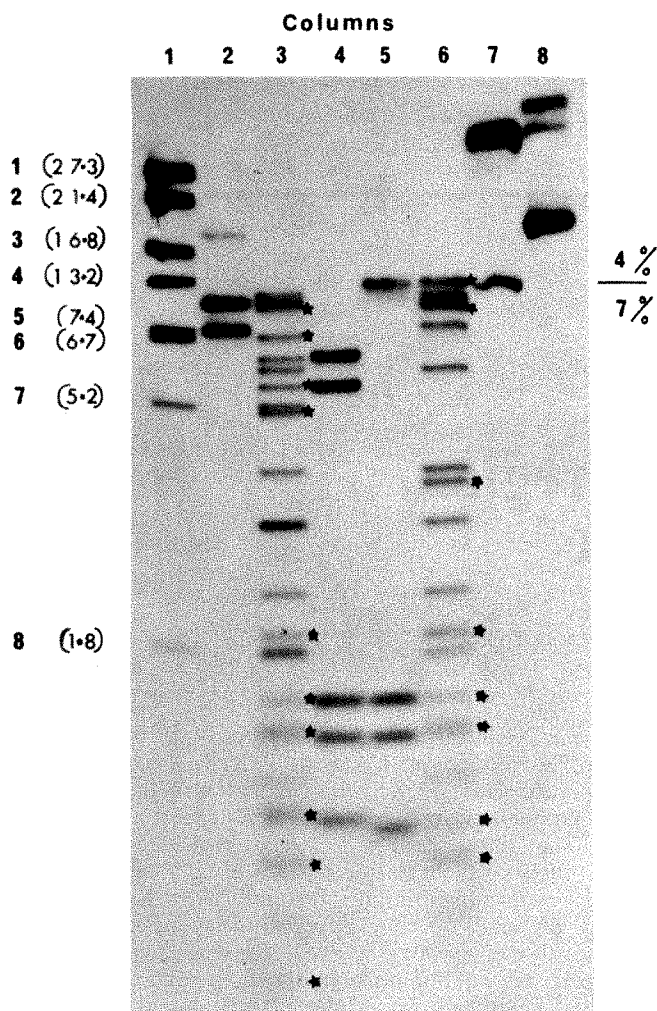
Fig. 2 Autoradiogram showing the position of restriction enzyme fragments from ^{32}P-labelled polyoma wild type (A-3 strain) DNA and D-50 DNA. Separation was accomplished by electrophoresis on a 4–7% acrylamide slab gel (20 × 40 cm) in Trisborate buffer (pH 8.3). The division between the two gel concentrations is indicated. 1, Fragments 1–8 obtained after cleavage of A-3 DNA with *Hpa*II; fragment sizes are given in parentheses (at left). The DNAs obtained from A-2 and A-3 polyoma virus stocks (both Pasadena large plaque strains) differ in the size of fragment *Hpa*II-5. This fragment is 7.7% genome length in A-2 (ref. 6) and 7.4% in A-3 DNA[6], the change occurring near the *Hpa*II 3–5 junction (B.E.G., unpublished). D-50 and related defective DNAs have the smaller *Hpa*II-5 fragment characteristic of the A-3 strain. 2, Two main fragments obtained by *Hpa*II cleavage of D-50 DNA. The single product (about 17% genome length) from a limited digest is seem to migrate slightly slower than *Hpa*II-3. 3, Fragments obtained after cleavage of a *Hpa*II-digested sample of A-3 DNA with *Hae*III. The position of fragments known to come from the region of the polyoma physical map which contains *Hpa*II-3, -4 and -5 (B.E.G., unpublished) are indicated (*). One of the fragments from *Hpa*II-5 is extremely small, and has been run off this gel. 4, Fragments obtained after cleavage of a *Hpa*II-digested sample of D-50 DNA with *Hae*III. 5, Fragments obtained after cleavage of D-50 DNA with *Hae*III. 6, Fragments obtained after cleavage of A-3 DNA with *Hae*III. The position of fragments known to come from the region of the polyoma physical map which contains *Hpa*II-3, -4 and -5 are indicated (*). 7, Fragments 1–3 obtained after cleavage of A-3 DNA with *Hha*I. One cleavage site is known to occur in *Hpa*II-2, another in *Hpa*II-6 and the third in *Hpa*II-5 (Fig. 4). The two large fragments (about 46% and 42%) are not clearly separated on this gel. 8, The single product (about 17% genome length) obtained after cleavage of D-50 DNA with *Hha*I. (Some partial and undigested material can also be seen.)

Most defective DNA is shorter than infectious polyoma DNA[1-3], contains only restricted portions of the viral genome, and in some cases may also contain host sequences

covalently linked to viral sequences[4]. Interest in the isolation of pure defective DNA populations has been stimulated by the belief that such molecules, arising as they do from situations in which selective pressures are being exerted within cells, would be extremely useful both for studying virus–cell interactions and the evolution of the viral genome. A number of different defective molecules have recently been cloned in the presence of infectious wild-type polyoma (helper) molecules[3]. Molecules about 50% (designated D-50) the size of infectious polyoma DNA were purified from one clonal isolate and were found by heteroduplex studies to be homogeneous in size and to contain a single region of homology to about 18% of the sequences in polyoma DNA[5].

This report describes the sequences present in D-50, shows that other defective molecules present in the D-50 preparation contain the same sequences, and relates these sequences to those found around the origin of DNA replication in wild-type polyoma DNA.

Characterisation of defective species

A population of DNA molecules isolated from cells infected with a virus stock containing both polyoma D-50 molecules and wild type helper virus was found to contain molecules corresponding to about 34, 51, 68, 85 and 102% the size of the wild type polyoma genome (Fig. 1). The 51% species was found to be identical with the D-50 DNA previously analysed by electron microscopy[5]. The designation D-50 for this species will therefore continue to be used in this report. Heteroduplex studies showed the region of homology between D-50 and wild type DNA molecules to begin on the latter about 16% from the *Escherichia coli* RI restriction enzyme (*Eco*RI) single cleavage site[5]. On the physical map of polyoma DNA this site can lie in either restriction fragment *Hpa*II-6 or *Hpa*II-4 (ref. 6), (Fig. 4). To locate the homology region on the polyoma physical map and define the D-50 and related defective species more precisely, a restriction enzyme analysis was performed.

Cleavage with restriction enzymes

The resistance of D-50 molecules to cleavage by *Eco*RI restriction enzyme was shown earlier[3,5]. The other defective species from the same virus stock were also resistant to *Eco*RI. Moreover, all the species were resistant to cleavage with enzymes from *Haemophilus influenzae*, *Hin*III and *Hin*II, enzymes which each cleave wild type polyoma DNA into two fragments[6,7]. Cleavage of the defective DNAs with *Hha*I (from *H. haemolyticus*) produced only one fragment (about 17% wild type genome length) which was not similar in size to any of the three fragments produced from wild type DNA (B.E.G., unpublished). With *Hpa*II, the defective species were cleaved to two fragments only. One fragment (about 7.4%) was electrophoretically indistinguishable from *Hpa*II-5 of the wild type A-3 strain[8]. The other (about 10%) did not correspond to any of the known wild type *Hpa*II fragments. Some of these results are shown in Fig. 2 (columns 2 and 8).

Treatment of D-50 with *Hpa*II in incomplete digestion conditions produced one predominant partial digestion product, which corresponded in length to about 17% of the wild-type genome (Fig. 2, column 2).

No partial digestion products less than 17% were detected, nor any about 20% in length. These results suggest that in these DNAs there is a basic subunit (with two *Hpa*II cleavage sites) which is tandemly repeated either two, three, four, five or six times to produce the different defective species.

Fine structure mapping with *Hae*III

Two separate studies were made to locate more precisely on the physical map of polyoma DNA the sequences present in the defective molecules. In one, the defective DNAs and
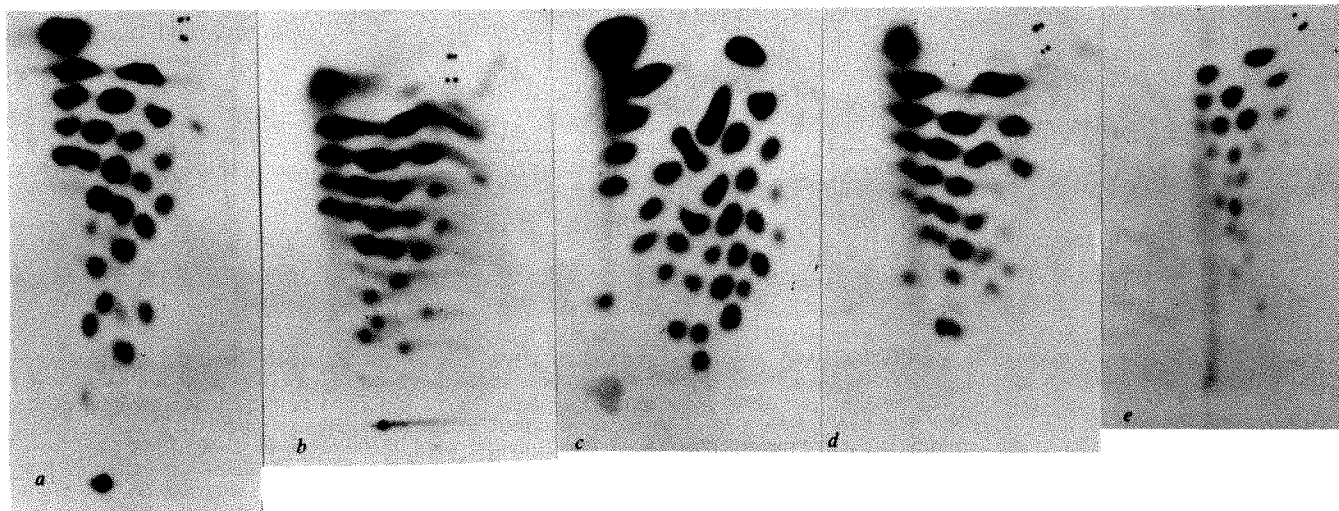
Fig. 3 Autoradiograms of the two-dimensional fractional separation of pyrimidine tracts of some depurinated ^{32}P-labelled *Hpa*II fragments from wild type polyoma (A-3 strain) DNA and D-50 DNA. Depurinations and separations were carried out as described previously[9]: the first dimensional separation (from right to left) was carried out by electrophoresis on cellulose acetate strips at *p*H 3.5, and the second dimensional separation (from bottom to top) by homochromatography using homomix C[18]. Fractionation of pyrimidine tracts from the *Hpa*II-5 fragment from A-3 DNA (*a*), and the 7.4% *Hpa*II fragment from D-50 DNA (*b*). *a* and *b* can be seen, at least qualitatively, to be identical. Fractionation of pyrimidine tracts from the *Hpa*II-3 fragment from A-3 DNA (*c*), the *Hpa*II-4 fragment from A-3 DNA (*e*) and the 10% *Hpa*II fragment from D-50 DNA (*d*). All pyrimidine tracts present in *d* can be found in either *c* or *e*, although many tracts present in *c* and *e*, or in the entire wild type genome (not shown), are not found in *d*. Three large oligonucleotides (including oligo (T$_{7 \text{ or } 8}$), marked* in *c*) are characteristic of *Hpa*II-3 (B.E.G., unpublished) and can be seen in the D-50 fragment (*d*). The isoplith C$_3$T$_3$, marked (*) in *e*, is present in high yield both in *Hpa*II-4 and *d*.

their *Hpa*II cleavage products were treated with a restriction enzyme from *H. aegyptius* (*Hae*III) which cleaves polyoma wild-type DNA into approximately 23 fragments. The relationship between the *Hae*III fragments and the *Hpa*II fragments (used to construct the physical map of the polyoma DNA[6]) are known (B.E.G., unpublished). Therefore, *Hae*III can be used as an effective probe into the composition of the defective molecules. The cleavage by *Hae*III alone (Fig. 2, column 5) of the defective species gave one large fragment (about 13% wild type genome length) together with three small fragments, two of which could be identified as coming from *Hpa*II-3 and one from *Hpa*II-5. In wild-type DNA, these three small fragments join to form a continuous sequence which includes the *Hpa*II 3-5 junction. Treatment of the defective DNAs with both *Hae*III and *Hpa*II (Fig. 2, column 4) showed that the larger (13%) *Hae*III product could be cleaved into two fragments. One was identical with the fragment from wild type strain A-3 *Hpa*II-5 (adjacent to the *Hpa*II 4-5 junction) and the other slightly smaller than the fragment from wild-type *Hpa*II-4 normally found at the 4-5 junction.

Since one end of the latter fragment must lie at the *Hpa*II 4-5 junction and the other at the *Hae*III site in *Hpa*II-3, this fragment contains sequences from both *Hpa*II-3 and -4.

Fine structure mapping by depurination

Depurination fingerprints[9] of D-50, and the 85 and 102% species were made and found to be identical, thus supporting the relationship between these species. They were considerably less complicated than a depurination fingerprint of wild-type polyoma DNA. Pyrimidine tracts characteristic of wild type *Hpa*II fragments 1, 2, 6, 7 and 8 were found to be absent (B.E.G., unpublished). No pyrimidine tracts were found in the defective species which were not present in wild-type polyoma DNA.

In a second study, a comparison of the depurination fingerprint of the smaller *Hpa*II fragment from D-50 (about 7.4% wild type genome length) with *Hpa*II-5 from wild type DNA showed the two to be identical. The fingerprint of the larger *Hpa*II fragment (about 10% genome length) showed pyrimidine tracts characteristic of both wild type fragments *Hpa*II-3 and -4 (Fig. 3).

All the results are consistent with the defective DNAs being composed of tandemly repeating 17% units made up of all the viral sequences from polyoma *Hpa*II-5 (wild type A-3 strain) and part of the sequences from *Hpa*II-3 and -4. The results of the study on D-50 are summarised on a physical map, relative to wild type DNA[6], in Fig. 4.

Conclusions

One of the spontaneously arising defective species from polyoma virus has been cloned[3] and is here studied in detail. It has been shown to be made up of molecules containing two- to sixfold tandem repeats of a segment of approximately 17% of viral DNA. Restriction enzyme analysis, especially with *Hae*III, has defined the viral sequences in the 17% subunit as extending from *Hpa*II-3 through *Hpa*II-5 into *Hpa*II-4 on the polyoma physical map[6]. One of the *Hae*III fragments from the defective molecules is not identical with any of the wild type *Hae*III fragments and represents a fusion of sequences from *Hpa*II-3 and *Hpa*II-4. From these data the exact location of the ends of the defective subunit cannot be precisely placed on the physical map. The correlation of these studies with the size of the homology region observed by Robberson and Fried[5] suggests that most if not all of the sequences in the subunit are viral. Assuming only viral sequences to be present and knowing the order and approximate size of the *Hae*III fragments of the wild type DNA in this region it can be estimated that of the sequences in the 17% subunit, 18–27% come from *Hpa*II-3 and 42–53% from *Hpa*II-4. The results on the size of the defective subunit and its position on the physical map of polyoma DNA are in good agreement with the previous heteroduplex studies[5].

The segment of viral DNA present in D-50 and related defectives contains two of the biologically interesting markers so far located on the physical map of polyoma
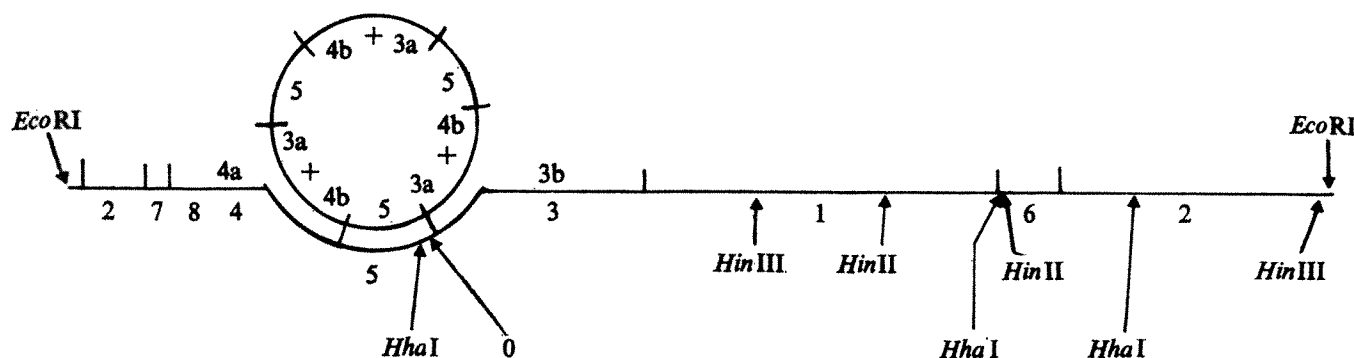
Fig. 4 Diagrammatic representation of the homology region between D-50 DNA (circle) and *Eco*RI-treated wild type polyoma DNA (linear). Homology begins $16\pm2\%$ from the left end of wild-type DNA and extends to about 34% from the same end[5]. Numbers 1–8 on linear map show the order of the eight *Hpa*II fragments of polyoma DNA[8], and 5 and 3a+4b (circle) the order of the two *Hpa*II fragments of D-50 (and the defective DNAs related to it, as described in the text). *Hpa*II fragments 5 (about 7.4%) and 3a+4b (about 10%) make up the subunit (about 17%) found tandemly repeated three times in D-50 DNA, and two, four, five and six times in the other defective DNA related to D-50. The approximate site of the origin of DNA replication (○) is indicated[6]; note that this site is repeated three times in D-50 DNA. The cleavage sites of three other restriction enzymes, *Hin*III[6], *Hin*II and *Hha*I (B.E.G. unpublished) are indicated on the physical map of polyoma DNA.

DNA. One is the origin of DNA replication, which has been mapped around the *Hpa*II 3–5 junction at 71 ± 3 map units[6,19]. The other contains the 5′ ends of the early and late stable RNAs (transcribed from different DNA strands) which have been mapped in *Hpa*II-5 (ref. 10). It is interesting to note that the regions in which viral DNA synthesis terminates[6,19] and where the 3′ ends of viral messengers have been mapped[10,] have not been detected in these defective DNAs.

Molecules containing multiple origins of DNA synthesis might be expected to have selective advantages for replication over those molecules with a single origin. When apparently defective-free virus is grown at high multiplicity for a single cycle, defectives similar to those described here arise with high frequency. Although the size of the basic subunit seems to be variable, in most cases it seems to contain *Hpa*II-5 and thus can be detected by greater than unimolar equivalents of this fragment in the DNA[8]. Another clonal isolate containing molecules about 47% (D-47)[5] of the viral genome seems to contain a repeating unit of a 16% segment of viral DNA which includes *Hpa*II-5 (B.E.G. and M.F., unpublished). Similar molecules with repeated segments of sequences which contain the origin of DNA replication have also been detected in SV-40 (ref. 12) and PML virus[11,20] passaged at high multiplicities. The detection of repeated viral sequences in *Eco*RI resistant defective molecules from uncloned polyoma DNA preparations derived from high multiplicity passage may also contain repeats in this region[13]. Note that the type of defective defined here seems to be only one from among several types of defective DNAs that arise after passage of polyoma virus at high multiplicity.

It is not clear at present how D-50 and related DNAs arise. There are a number of mechanisms by which these defective DNAs can be generated. One possibility is that they are formed by errors in replication[8]. The study of other cloned defectives with basic subunits of different sizes will help clarify their origin. For instance, if the midpoint of all the different defective subunits were the same it would suggest that DNA synthesis proceeds bidirectionally at equal rates before the subunit is excised and ligated. On the other hand, if one end of the defective basic subunit were always at the same position on the physical map whereas the other end varied in position then unidirection replication for various lengths before excision and ligation might be suggested.

In this study, a correlation between the quantity of any species of defective DNA in the clonal isolate and the number of tandem repeats of the 17% subunit in that species has been observed (Fig. 1). Two explanations may

account for the greater abundance of the larger defective species. One would be the greater selective advantage for replication available to molecules with more origins of DNA synthesis. The other would be the possible difficulty of stable encapsidation (necessary for continued passage of the particular species) of the smaller DNA molecules.

The defective molecules containing multiple tandem sequence repeats are presumably derived from a common precursor of about 17% of the polyoma genome. We have not been successful in isolating this postulated small precursor molecule, but it would not be expected to be present in great quantities for reasons given above. The DNA isolation procedure used would also tend to discriminate against such a low molecular weight molecule. One of the interesting results from the present study is, however, the apparent uniformity of the repeating subunit. The results from a number of experiments suggest that there is little or no variation in the size of the *Hpa*II 7.4% fragment and if there is any variation in the size of the 10% *Hpa*II fragment, it is outside the scope of detection of the methods used. This suggests that D-50 and related molecules are derived from the subunit by recombination events.

Further, it is interesting to note that DNA derived from virus stocks produced from purified D-50 molecules and added helper DNA generated all the species of the multiple repeat defectives found in the parental population (Fig. 1). This generation of both larger and smaller related defective molecules from the D-50 species suggests that intramolecular and possibly intermolecular recombination processes can occur. Moreover, in the DNA produced from virus stocks made from the purified D-50 molecules the D-50 species is seen to be present in greater yields than the 68% species (Fig. 1*a* and *b*), whereas in the parental population the reverse is true (Fig. 1*c*). This seems to mitigate against the rolling circle model of DNA replication[22] as a mechanism for the generation of the related defectives, as this type of replication would not show any preference for the production of D-50 species over the 68% species.

We thank Drs R. Roberts, R. Old and R. Kamen for restriction enzymes, and Mrs M. Griffiths for assistance.

1 Thorne, H. V., Evans, J., and Warden, D., *Nature*, 219, 728-730 (1968).
2 Blackstein, M. E., Stanners, C. P., and Farmilo, A. J., *J. molec. Biol.*, 42, 301-313 (1969).
3 Fried, M., *J. Virol.*, 13, 939-946 (1974).
4 Lavi, S., and Winocour, E., *Virology*, 57, 296-299 (1974).
5 Robberson, D. L., and Fried, M., *Proc. natn. Acad. Sci. U.S.A.*, 71, 3491-3501 (1974).
6 Griffin, B. E., Fried, M., and Cowie, A., *Proc. natn. Acad. Sci. U.S.A.*, 71, 2077-2081 (1974).
7 Chen, M. C. Y., Chang, K. S. S., and Salzman, N. P., *J. Virol.*, 15, 191-198 (1975).

[8] Fried, M., Griffin, B. E., Lund, E., and Robberson, D. L., *Cold Spring Harb. Symp. quant. Biol.*, 39, 45-52 (1974).
[9] Ling, V., *J. molec. Biol*, 64, 87-102 (1972).
[10] Kamen, R., Lindstrom, D M., Shure, H., and Old, R. W, *Cold Spring Harb. Symp. quant Biol.*, 39, 187-198 (1974).
[11] Khoury, G., *et al*, *J. molec. Biol*, 87, 289-301 (1974).
[12] Brockman, W. W, Lee, T N. H., and Nathans, D., *Cold Spring Harb Symp quant Bioll.*, 39, 119-127 (1974).
[13] Folk, W. R, and Wang, H-C C., *Virology*, 61, 140-155 (1974).
[14] Sharp, P. A, Sugden, B, and Sambrook, J., *Biochemistry*, 12, 3055-3063 (1973).
[15] Hirt, B., *J molec. Biol.*, 26, 365-369 (1967)

[16] Radloff, R., Bauer, W., and Vinograd, J., *Proc. natn. Acad. Sci. U.S.A.*, 57, 1514-1521 (1967).
[17] Galibert, F, Sedat, J. and Ziff, E., *J. molec., Biol.*, 87, 377-407 (1974).
[18] Brownlee, G G., and Sanger, F., *Eur. J Biochem.*, 11, 395-399 (1969).
[19] Crawford, L. V., Robbins, A. K., and Nicklin, P M, *J. gen. Virol.*, 25, 133-142 (1974)
[20] Davoli, D., and Fareed, G C., *Nature*, 251, 153-155 (1974).
[21] Cuzin, F. M., Vogt, M., Dieckmann, M., and Berg, P., *J. molec. Biol.*, 47, 317-333 (1970).
[22] Gilbert, W., and Dressler, D., *Cold Spring Harb. Symp. quant. Biol.*, 33, 473-484 (1968).

Implications for theories of anaesthesia of antagonism between anaesthetic and non-anaesthetic steroids

C. D. Richards & T. R. Hesketh

National Institute for Medical Research, Mill Hill, London NW7 1AA, and Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK

The non-anaesthetic steroid $\Delta16$-alphaxalone antagonises the depressant action of the anaesthetic steroid alphaxalone on synaptic transmission. This antagonism is not predicted by classical theories of anaesthesia and therefore poses fundamental questions about the molecular basis of anaesthesia.

IT is now widely accepted that there is a good correlation between the anaesthetic potency of a substance and its lipid solubility[1-4]. This correlation has led to a number of hypotheses of anaesthetic action, notably the Meyer–Overton rule of lipid solubility[5] and the critical volume hypothesis of Mullins[6]. These hypotheses state that anaesthesia occurs whenever a certain critical concentration or a critical molar volume of an inert substance is achieved in the neuronal membranes. These theories have a serious weakness, however, in that they concentrate attention on substances that are both lipid-soluble and anaesthetic, and ignore those that are lipid-soluble but have no anaesthetic properties.

Some steroids possess anaesthetic properties whereas others do not[7]. Systematic examination of some 5α-pregnanes has shown that steroids having the same functional groups could be either anaesthetic or non-anaesthetic according to their precise structure[8,9]. These observations are not easily reconciled with the hypotheses of anaesthetic action based on lipid solubility, as the steroids are themselves lipids. It could be argued that the non-anaesthetic steroids fail to produce general anaesthesia simply because they do not reach the brain in sufficient quantity to cause anaesthesia. It follows from this argument that, if we could apply 'non-anaesthetic' steroids directly to the nerve cells of the brain, we should be able to reveal their anaesthetic properties.

One preparation suitable for testing this idea is the isolated olfactory cortex of the guinea pig[10,11]. A number of general anaesthetics depress excitatory synaptic transmission in this preparation at concentrations likely to be found in the brain during anaesthesia[12-15]. If we could show that anaesthetic steroids also depressed excitatory synaptic transmission in this preparation, and that non-anaesthetic steroids did not, then we could conclude that either the non-anaesthetic steroids cannot penetrate synaptic membranes or that they can, but fail to alter normal membrane function. In either event some factor in addition to lipid solubility would be required to explain such results.

In the work presented here, we have examined the pharmacological action of two closely related steroids on synaptic transmission in the olfactory cortex. One of the compounds,

3α-hydroxy-5α-pregnane-11,20-dione (alphaxalone) is a powerful anaesthetic and is the main active component in the clinically used anaesthetic Althesin. The other, 3α-hydroxy-5α-pregnan-16-ene-11,20-dione ($\Delta16$-alphaxalone) is non-anaesthetic[9]. The structures of these two substances (Fig. 1) differ only by a double bond in the D ring of the steroid nucleus.

Isolated preparations of guinea pig olfactory cortex have been shown to generate stable and characteristic field potentials in response to electrical stimulation of the lateral olfactory tract (LOT)[11-15]. If a recording electrode is placed on the pial surface of the prepiriform cortex close to the LOT and a pulse is subsequently applied to the anterior end of the LOT, a complex wave form can be recorded (see Fig. 2). This comprises an initial wave—the compound action potential caused by activity in the LOT fibres—followed by a negative wave (the population excitatory postsynaptic potential, or population e.p.s.p.) which has been identified as the field potential arising from the synchronous depolarisation of the apical dendrites of many of the cortical neurones. If the population e.p.s.p. is sufficiently large, a number of positive peaks may be superimposed on it. These peaks reflect the synchronous discharge of the cortical cells in response to the population e.p.s.p. and are called population spikes[17] (see Fig. 2). (If the recording electrode is placed in the pyramidal cell layer instead of on the cortical surface, a similar potential can be recorded, but of reversed polarity[18] (Fig. 3).) The amplitude of the population e.p.s.p. is proportional to the number of active synapses and to the degree of depolarisation of each postsynaptic site; the area of the population spikes is proportional to the number of discharging cortical cells. In this paper we have taken the amplitude

Fig. 1 Structural formulae of steroids used. *a*, Alphaxalone; *b*, $\Delta16$-alphaxalone.
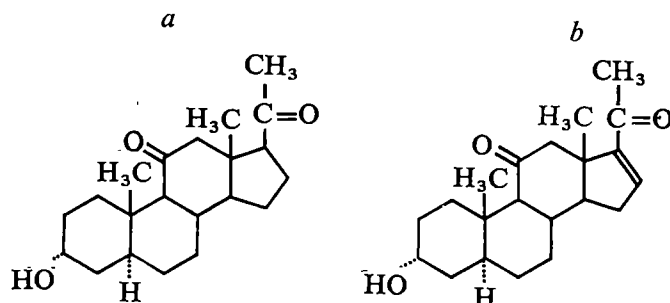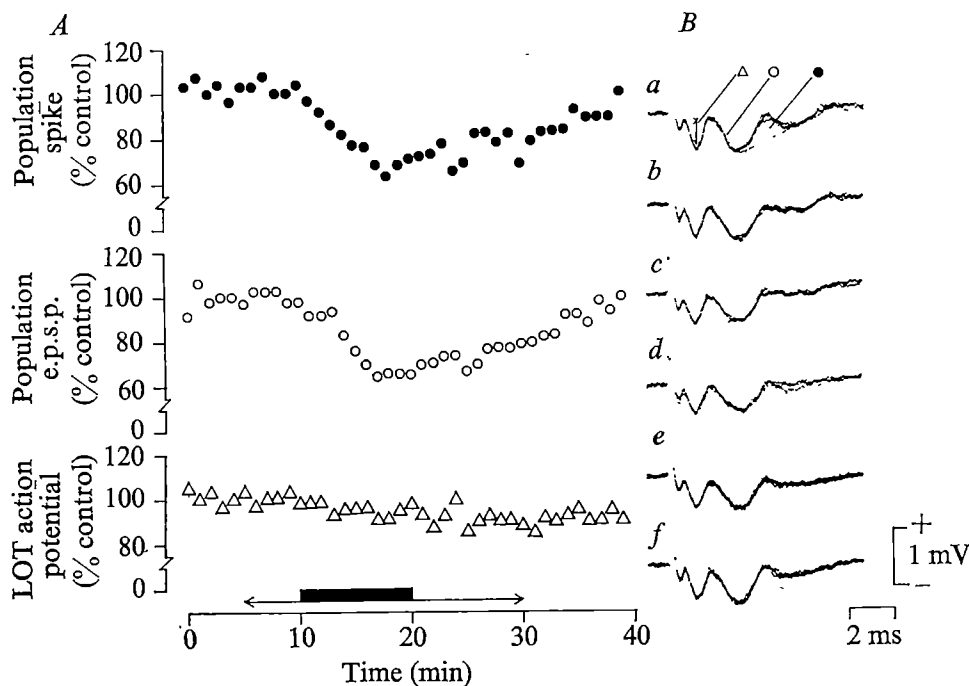
Fig. 2 Depressant action of alphax-alone on the evoked potentials of the olfactory cortex. *A*, Time course of experiment. Arrowed line under black bar indicates period during which CSF containing liposomes was superfusing the preparation. Black bar indicates period of exposure to alphaxalone (5×10^{-5} M) *B*, Sample records of four to six superimposed sweeps taken at various times during the experiment. *a*, Control, *b*, 6 min in liposomes; *c*, 5 min in alphaxalone; *d*, 10 min in alphaxalone; *e*, 10 min after alphax-alone; *f*, recovery. Alphaxalone was administered as the steroid component of liposomes. Egg yolk lecithin was dissolved in chloroform–methanol (2:1 v/v) together with the steroid in a molar ratio of 1:1. The solutions were evaporated to dryness under nitrogen and kept under vacuum (10^{-3} mmHg) for 1–4 h to remove residual traces of solvent. Artificial CSF (refs 12–14) was then added to yield a final concentration of 50 μM phospholipid. Solutions at 4 °C were then subjected to ultrasonic dispersion for 2–5 min using an MSE probe sonicator to suspend the phospholipid as small vesicles.

of the population e.p.s.p. at a fixed latency from the stimulus as our chief measure of postsynaptic activity[12–15].

As steroids are very hydrophobic substances, they are very sparingly soluble in water. Clinically, they are administered as micelles with Cremaphor EL (a polyoxyethylated castor oil) as the carrier[16] but Cremaphor was toxic to our isolated preparations. To overcome this, we applied alphaxalone as the steroid component of lecithin vesicles (liposomes) suspended in the artificial cerebrospinal fluid (CSF) that superfused the isolated cortical tissue. This method of applying the steroids has proved quite satisfactory.

When liposomes containing cholesterol, or lacking a steroid component, were added to the artificial CSF that superfused the isolated cortical tissue, little change was seen in the evoked potentials (Figs 2 and 3). If the superfusing artificial CSF containing lecithin liposomes was changed for one containing liposomes with alphaxalone in a 1:1 molar ratio with the phospholipid (to give a final concentration of 5×10^{-5} M alphaxalone in the artificial CSF) then the population e.p.s.p. and population spikes became depressed but the LOT compound action potential was not affected (Fig. 2). Provided the tissue was not exposed to the alphaxalone for long periods (greater than 20 min) the depression of the population e.p.s.p. and population spike rapidly reversed when artificial CSF containing lecithin liposomes replaced that containing alphaxalone-loaded liposomes. Thus, alphaxalone depressed the population e.p.s.p. and population spikes without blocking nerve impulse conduction in the afferent LOT fibres. The depression of the population e.p.s.p., however, produced by a given dose of alphaxalone varied rather widely, in the range 25–100% for a concentration of 5×10^{-5} M in the CSF, although it generally lay between 30 and 60% (Table 1). As with other general anaesthetics[12–15] this depression occurred at concentrations of alphaxalone likely to be found in the brain during anaesthesia. (An anaesthetic dose of alphaxalone has been found to be approximately 3 mg kg⁻¹ for a number of species[16]. As the anaesthetic must reach the brain cells by way of the extracellular fluid which accounts for 20–30% of the total body weight[19], 3 mg kg⁻¹ alphaxalone given intravenously will have a maximum concentration in the extracellular space of 3×10^{-5} to 5×10^{-5} M (ref. 12).)

If the liposomes superfusing the tissue contained not alphaxalone but Δ16-alphaxalone, a very much smaller depression of

the population e.p.s.p. of about 10–15% was seen (Table 1). Furthermore, increasing the duration of the superfusion by Δ16-alphaxalone beyond the normal 10 min period (up to a maximum of 45 min) did not further increase the depression of the population e.p.s.p.

These results correlate well with the effects of these steroids *in vivo;* the anaesthetic steroid alphaxalone depresses the population e.p.s.p. by 30–60%, whereas the non-anaesthetic steroid Δ16-alphaxalone has little depressant action. These results suggest that both steroids can penetrate the synaptic membranes, as they both depress the population e.p.s.p., but, unlike alphaxalone, Δ16-alphaxalone is not able to disrupt synaptic transmission to any great extent.

Antagonism of depressant action of alphaxalone by Δ16-alphaxalone

After an experiment in which the depressant action of Δ16-alphaxalone had been tested, we exposed the same preparation to alphaxalone and discovered that alphaxalone had only a slight depressant action. The preparation was left in artificial CSF for 3 h and was then exposed again to alphaxalone. After this time the preparation regained its sensitivity to alphaxalone (Fig. 3). This antagonism of the depressant action of alphaxalone by Δ16-alphaxalone has now been confirmed in ten separate experiments using two different samples of alphaxalone and Δ16-alphaxalone, and three different samples of lecithin (Table 1). Of the ten experiments, four showed almost complete reversal of the Δ16-alphaxalone block 1–3 h after exposure to Δ16-alphaxalone, two showed a partial reversal and in the remaining four experiments, the depressant action of alphaxalone was blocked throughout the experiment (up to 3 h).

If a preparation had first been exposed to alphaxalone before treatment with Δ16-alphaxalone, subsequent exposures to alphaxalone generally caused the same depression of the e.p.s.p. as that seen initially. To reveal the antagonism of the depressant action of alphaxalone by Δ16-alphaxalone it was necessary to expose the tissue to Δ16-alphaxalone—before making any test for the depressant action of alphaxalone. For this reason the depressant action of the alphaxalone-containing liposomes was checked in a parallel experiment on a preparation not pretreated with Δ16-alphaxalone. Both the control experiments and those demonstrating the antagonism were performed on the same day to eliminate variation in the release of alphaxalone
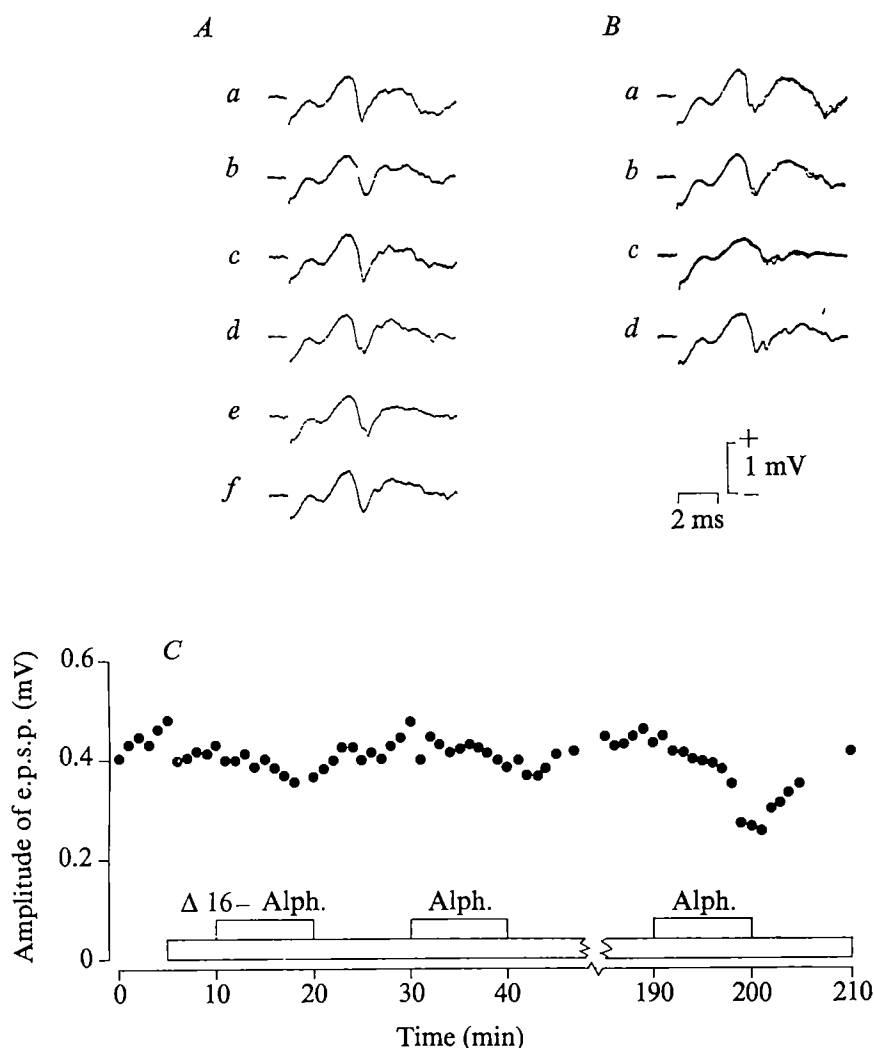
Fig. 3 Action of Δ16-alphaxalone on evoked potentials of olfactory cortex. *A* and *B*, Sample records taken from the cell body region of the slice at various times during the experiment shown below. *A*, Records taken in the first 45 min of the experiment. *a*, Control; *b*, Δ16-alphaxalone; *c*, control 2; *d*, 5 min in alphaxalone; *e*, 10 min in alphaxalone; *f*, recovery. *B*, Records taken after 185 min. *a*, Control 3; *b*, 5 min in alphaxalone; *c*, 10 min in alphaxaone; *d*, recovery. *C*, Time course of the experiment. Δ16-alphaxalone (5 × 10⁻⁵ M) had little depressant effect. Initial exposure to alphaxalone (5 × 10⁻⁵ M) showed only slight depressant activity but a subsequent exposure to alphaxalone (approximately 3 h after the exposure to Δ16-alphaxalone) markedly depressed the population e.p.s.p. Lecithin (10⁻⁴ M) in CSF after 5 min for duration of experiment

from liposomes as a result of ageing or the formation of lysolecithin. One example of these paired experiments can be seen in Fig. 4.

Nature of the antagonism

The antagonism of the depressant action of alphaxalone by Δ16-alphaxalone could be the result of one of two processess either Δ16-alphaxalone prevents the release of alphaxalone from the liposomes (that is, it is an artefact of the method of application) or it antagonises the depressant action of alphaxalone on some process involved in synaptic transmission.

As the antagonistic effects of Δ16-alphaxalone on the depression of the e.p.s.p. by alphaxalone could persist for several hours, it seems unlikely that small amounts of Δ16-alphaxalone were merely reducing the release of alphaxalone from the phospholipid vesicles. This argument is further supported by the observation that, following a short exposure (5 min) to Δ16-alphaxalone, one preparation did not exhibit resistance to the

depressant action of alphaxalone. Moreover, the antagonism did not develop if a preparation had first been exposed to alphaxalone before treatment with Δ16-alphaxalone. These observations strongly suggest that the antagonism is a genuine pharmacological event.

Given that Δ16-alphaxalone antagonises the depressant action of alphaxalone by some biological mechanism, there are four ways in which this could be brought about. First, Δ16-alphaxalone could prevent alphaxalone penetrating the synaptic membranes. Second, Δ16-alphaxalone could be acting as a competitive antagonist at some specific site or sites. Third, as synaptic transmission is a multistage process, Δ16-alphaxalone and alphaxalone could act at different sites but have opposing actions which tend to cancel. Fourth, Δ16-alphaxalone could desensitise the preparation to alphaxalone.

Of these four possibilities, the first two seem the most plausible but we have no evidence that enables us to distinguish between them. The third possibility seems unlikely because

Table 1 Depression of population e p.s p. at the end of a 10-min exposure to 50 μM alphaxalone or 50 μM Δ16-alphaxalone

Drug	Pretreatment	Depression of population e.p.s.p. (% control)			No. of experiments
		Mean	± s.e.m.	Range	
Δ16-alphaxalone	None	14.8	± 2.5%	0–25%	9
Alphaxalone	None	45.7	± 5.7%	25–100%	12
Alphaxalone (10 min after pretreatment)	(20 min in Δ16-alphaxalone)	9.4	± 3.26%	0–35%	10
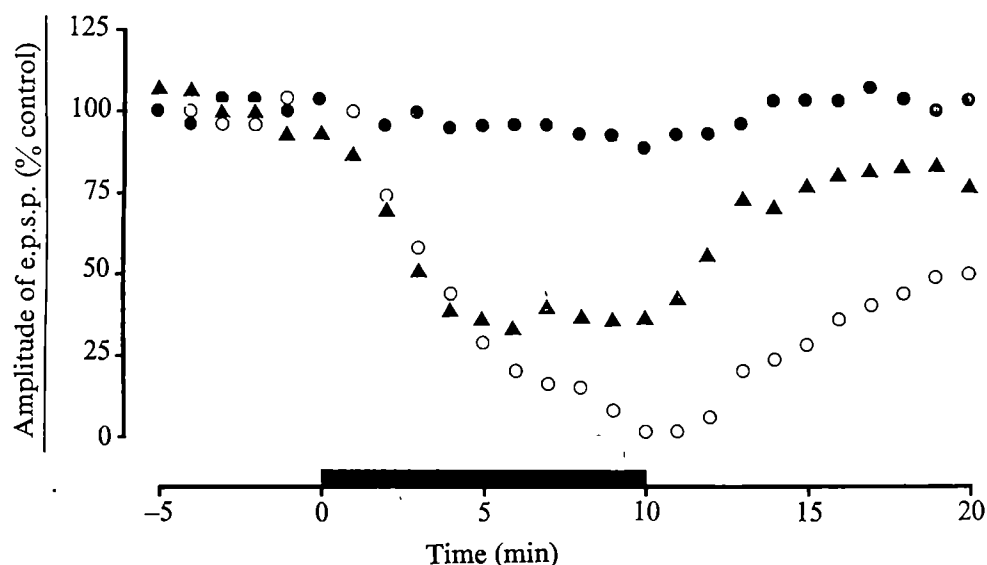
Fig. 4 Example of blocking of alphaxalone action by Δ16-alphax-alone and its subsequent recovery compared with the depression of the e.p.s.p. produced in a preparation not pretreated with Δ16-alphaxalone. O, Depression produced in an untreated preparation; ●, block of alphaxalone-induced depression 12 min after exposure to Δ16-alphaxalone; ▲, recovery of sensitivity to alphaxalone 160 min after exposure to Δ16-alphaxalone. Bar, alphaxalone $(5 \times 10^{-5}$ M) exposure.

Δ16-alphaxalone and alphaxalone show qualitatively similar effects on the synaptic potentials. Furthermore, the antagonism outlasts the depressant action of Δ16-alphaxalone (Fig. 3). The fourth possibility is also unlikely as Δ16-alphaxalone does not antagonise the depressant action of alphaxalone in preparations which had been exposed to alphaxalone before treatment with Δ16-alphaxalone. Further indirect evidence against a desensitisation hypothesis comes from experiments on mice, in which it has been shown that successive injections of alphaxalone result in a slight increase in the sleep time resulting from a given dose of anaesthetic[20].

It is possible that the antagonism between these steroids reflects the existence of specific anaesthetic receptors for steroids, especially as the structure–activity relationships for anaesthetic potency are so well defined[8,9]. No firm answer can however, be given at present, but it should be noted that both 5α-and 5β-pregnanes may be anaesthetic in spite of the marked difference in the shape of the steroid nucleus in these two series. Furthermore, steroids are very lipid-soluble and show some of the nonspecific membrane effects of other general anaesthetics[4]— for example, they protect erythrocytes from lysis[21], presumably by expanding the membranes[4].

Implications for theories of anaesthesia

Hypotheses of anaesthetic action based on simple solubility models, such as the Meyer–Overton lipid solubility rule of anaesthesia[5] or the critical volume hypothesis of Mullins[6], imply that all anaesthetics act in the same region. This has led to the formulation of the unitary hypothesis of anaesthetic action, in which it is asssumed that general anaesthetics produce their effects by simple physicochemical interactions with hydrophobic regions of the cellular membranes, and not by interaction with specific sites[22]. It is implicit in all three hypotheses that the effects of two lipophilic compounds should be synergistic, not antagonistic[23], and this has indeed been shown for several pairs of compounds[24,25]. One striking example is the synergism of tetrahydrocannabinol and diethyl ether. Although tetrahydrocannabinol is highly lipophilic it is not an anaesthetic, yet when given intravenously to mice it reduces the amount of ether required to abolish the righting reflex[26].

One hypothesis of anaesthetic action does predict the existence of antagonism between different classes of anaesthetic. This is the degenerate perturbation hypothesis proposed by Metcalfe *et al.*[27] to account for the local anaesthetic action of a wide variety of organic compounds. This hypothesis is based on the opposing actions, on model membrane systems, of small molecules such as benzyl alcohol and large rigid molecules, such as androstane; benzyl alcohol increases and androstane decreases the fluidity of phospholipid bilayers[28-30]. Although

clinical concentrations of some general anaesthetics (halothane and methoxyflurane) have been shown to fluidise phospholipid bilayers[31], preliminary results with electron spin resonance techniques have shown no change in membrane fluidity when either alphaxalone or Δ16-alphaxalone are added to a phospholipid bilayer (T.R.H., unpublished). This suggests that the antagonism of the depressant action of alphaxalone by Δ16-alphaxalone is not mediated by generalised perturbations of membrane structure and presumably depends on more specific interactions within the synaptic membranes.

The antagonism of the effects of alphaxalone by Δ16-alphaxalone cannot be accommodated by any of the hypotheses of anaesthetic action mentioned above and we must, therefore, conclude that they do not apply to anaesthesia induced by steroids. It remains to be seen whether substances other than Δ16-alphaxalone can antagonise the depressant action of alphaxalone and whether Δ16-alphaxalone can antagonise the action of other general anaesthetics on synaptic transmission.

We thank our colleagues for reading and commenting on the manuscript, and Mr B. Davis and Dr G. H. Phillipps of Glaxo Research Ltd for the steroids and for discussion.

1 Eger, E. I., Lundgren, C., Miller, S. L , and Stevens, W. C., *Anesthesiology*, 30, 129 (1969).
2 Lever, M. J., Miller, K. W., Paton, W. D. M., and Smith, E B., *Nature*, 231, 368 (1971).
3 Miller, K. W , Paton, W. D. M , Smith, E. B , and Smith, R A., *Anesthesiology*, 36, 339 (1972).
4 Seeman, P., *Pharmac. Rev.*, 24, 583 (1972)
5 Meyer, K. H., *Trans. Faraday Soc*, 33, 1062 (1937).
6 Mullins, L. J., *Chem. Rev*, 54, 289 (1954)
7 Selye, H., *Proc Soc. exp. Biol. Med.*, 46, 116 (1941).
8 Atkinson, R. M., Davis, B , Pratt, M A., Sharpe, H. M., and Tomich, E. G , *J. med. Chem.*, 8, 426 (1965).
9 Phillipps, G. H., in *Molecular Mechanisms in General Anaesthesia*, ch 3 (Churchill–Livingstone, London, 1974)
10 Yamamoto, C , and McIlwain, H., *J Neurochem* , 13, 1333 (1966)
11 Richards, C. D , and Sercombe, R., *J Physiol , Lond*, 197, 667 (1968).
12 Richards, C. D , *J. Physiol., Lond.*, 227, 749 (1972)
13 Richards, C. D., *J Physiol., Lond.*, 233, 439 (1973).
14 Richards,C.D., Russell, W J., and Smaje, J C.,*J. Physiol.,Lond* ,248,121,(1975)
15 Richards, C D., in *MolecularMechanisms in General Anaesthesia*, ch 6(Churchill–Livingstone, London, 1974)
16 Child, K. J., *et al* , *Br. J Anaesth.*, 43, 2 (1971)
17 Richards, C. D., and ter Keurs, W. J., *Brain Res* , 26, 446 (1971)
18 Richards, C. D , and Sercombe, R., *J. Physiol , Lond* , 211, 571 (1970)
19 Spector, W. S. (ed), *Handbook of Biological Data*, 340 (Saunders, Philadelphia, 1956)
20 Davis, B., and Pearce, D. B , *Postgrad. med. J.*, 48, suppl. 2, 13 (1972)
21 Seeman, P., *Biochem. Pharmac.*, 15, 1632 (1966).
22 Hill, M. W , in *Molecular Mechanisms in General Anaesthesia*, ch. 8 (Churchill–Livingstone, London, 1974).
23 Smith, E B., in *Molecular Mechanisms in General Anaesthesia*, ch. 7 (Churchill–Livingstone, London, 1974).
24 Paton, W. D. M , and Temple, D , *Br. J. Pharmac* , 44, 346P (1972)
25 Miller, R. D., *et al.*, *Anaesthesiology*, 31, 301 (1969).
26 Paton, W D. M., in *Molecular Mechanisms in General Anaesthesia*, ch 4, 57 (Churchill–Livingstone, London, 1974).
27 Metcalfe, J C., Hoult, J. R. S., and Colley, C. M , in *Molecular Mechanisms in General Anaesthesia*, ch. 9 (Churchill–Livingstone, London, 1974).
28 Hubbell, W. L., Metcalfe, J C., Metcalfe, S M , and McConnell, H. M., *Biochim biophys. Acta*, 219, 415 (1970).
29 Lee, A G , Birdsall, N. J. M., and Metcalfe, J C., *Biochemistry*, 12, 1650 (1973).
30 Metcalfe, J C., Seeman, P , and Burgen, A S. V., *Molec Pharmac.*, 4, 87 (1968).
31 Trudell, S. R., Hubbell, W. L., and Cohen, E. N , *Biochim. biophys. Acta*, 291, 321 (1973).

Isolation of polar insertion mutants and the direction of transcription of ribosomal protein genes in *E. coli*

S. R. Jaskunas, L. Lindahl & M. Nomura

Institute for Enzyme Research, Departments of Genetics and Biochemistry, University of Wisconsin, Wisconsin 53706

Transcription of ribosomal protein genes in the str-spc *region in* Escherichia coli *seems to be exclusively anti-clockwise. Insertion mutations in this region inactivate many but not all distal ribosomal protein genes. Thus, there seems to be more than one transcriptional unit for the ribosomal protein genes in this region.*

MANY ribosomal protein (r-protein) genes in *Escherichia coli* are clustered at the *str-spc* region of the chromosome (see ref. 1 for review). Little is known about the organisation of these genes, however, or the mechanism of the regulation of their expression. Recent isolation of λ transducing phages carrying many r-protein genes from this region[2] has made it possible to study these problems in detail. Here we report the isolation of insertion mutations of the transducing phage λ*spc*1 that inactivate the expression of many, but not all, of the r-protein genes carried by this phage. In this connection, we have reinvestigated the direction of transcription of r-protein genes in the *str-spc* region. We have found that, contrary to the previous suggestion[3], the direction of transcription is anti-clockwise on the *E. coli* genetic map. The inactivated genes in our mutants are distal to the sites of the insertion mutations, and may belong to a single transcriptional unit.

Insertion mutations affecting r-protein genes

The λ*spc*1 phage carries a *spc*^{s} gene as well as *aroE*$^+$ and *trkA*$^+$ genes[2]. Bacterial strains carrying the *spc*^{r} allele become spectinomycin-sensitive (Spc-S) when they are lysogenised with λ*spc*1, because *spc*^{s} is dominant over the *spc*^{r} allele[21]. The lysogen from which we isolated Spc-R mutants had the following relevant chromosomal genetic markers: *KdpABC5*, *trkA*401, *spc*^{r}, *str*^{r}, *fus*^{r}, *recA*; and carried λ*spc*1 and a helper phage λ*c*1857*S*7. Thus this strain is diploid for the *spc* gene (*spc*^{s}/*spc*^{r}) and is phenotypically Spc-S. The selection of Spc-R mutants was done on tryptone-yeast extract agar plates containing Spc (100 μg ml^{-1}). This medium has a low concentration of potassium and does not enable growth of *trkA* mutants. Thus Spc-R colonies selected on this medium must have retained the *trkA*$^+$ gene carried by the λ*spc*1 phage. The Spc-R phenotype presumably resulted from some mutation that inactivated or altered the *spc*^{s} gene on the phage.

Transducing phages were then isolated from these Spc-R mutants and characterised. Out of 20 independently isolated Spc-R colonies the transducing phage could be recovered from 18, and 14 of them carried an insertion of a small piece of DNA in the phage (see below). Thus the inactivation of the *spc*^{s} gene in these 14 mutants seemed to be a result of an insertion. Two such mutants, λ*spc*1–I15 and λ*spc*1–I16, were studied in detail.

To examine the expression of other r-protein genes carried

by these mutant phages, we have measured the stimulation of r-protein synthesis in ultraviolet-irradiated *E. coli* cells after infection by the mutant phages and compared the stimulation with that caused by the parental transducing phage λ*spc*1 (Table 1).

The parent λ*spc*1 stimulates the synthesis of about 20 r-proteins (ref. 2 and Table 1). The strong stimulation of the synthesis of proteins S4, S11, S13 and L17 (see group I in Table 1) was also observed following infection by the mutant phages λ*spc*1–I15 and λ*spc*1–I16. The stimulation of the synthesis of many other r-proteins including S5, which is coded for by the *spc*^{s} gene (group II in Table 1), was, however, greatly reduced or abolished with λ*spc*1–I16. The reduction in the stimulation of the synthesis of these proteins was in general smaller with λ*spc*1–I15, but was still significant compared with the group I proteins. There are some proteins (group III in Table 1), whose synthesis was weakly stimulated by λ*spc*1 phage, or which were difficult to classify into group I or II with respect to the susceptibility of their synthesis to the insertion mutations (see the legend to Table 1).

It should be noted that the effect of the I15 insertion on the stimulation of the synthesis of group II proteins is not uniform. In particular, the stimulation of the synthesis of L24 was almost completely abolished by the I15 insertion. It is possible that the I15 insertion is within the gene for L24.

We have also done similar experiments with several other insertion mutants of λ*spc*1. As described below, the size of inserted DNA in λ*spc*1–I15 is different from that in λ*spc*1–I16. All other insertion mutants could be classified into two classes with respect to the size of inserted DNA, one about the same as that of I15, and the other about the same as that of I16. It was observed that other insertion mutants of "I16 type", like I16 itself, showed marked effects on the synthesis of the group II proteins, but not on that of the group I proteins. Conversely, the effects observed by mutants of "I15 type" were in general similar to those observed with λ*spc*1–I15: that is, partial inactivation of many or all of group II protein genes and weak or no inactivation of group I protein genes.

These data indicate that there are at least two classes of r-protein genes on λ*spc*1 whose expression is controlled separately. The insertion mutations studied here seem to appreciably affect one class (group II) but not the other(s) (group I). The weak inactivation found for group I proteins and the difference between the two experiments reported in Table 1 may be a result of slight differences in the multiplicity of infection (see legend to Table 1).

It should be noted that we have proved only that the structural genes for S4, S5, S8, S11, S13 and S14 are carried by λ*spc*1 since these proteins are made in our DNA-dependent *in vitro* protein synthesising system using λ*spc*1 DNA as template[2]. It is highly likely, however, that the structural genes for most of the other proteins in groups I and II are also carried by

Table 1 Stimulation of the synthesis of individual r-proteins by λspc1, λspc1–I15 (I15) and λspc1–I16 (I16)

Proteins	³H/¹⁴C ratio				% Decrease in stimulation	
	λS7	λspc1	115	116	115	116
Group I						
S4	0.03	6.31	4.78	3.99	24 (15)*	37 (5)*
S11+S9†	0.08	1 24	1.04	0 84	14 (15)	34 (20)
S13	0.12	7.20	5.31	4.53	27 (20)	38 (18)
L17	0.06	11.68	8.25	7 97	29 (16)	32 (5)
Group II						
S5	0.05	3.60	1 80	0.52	51 (49)	87 (66)
S8	0.04	1.68	0.63	0.11	64 (64)	96 (74)
S14	0.13	3.08	1.32	0.40	60 (50)	91 (75)
L5	0.04	2.50	0.94	0.14	63 (68)	96 (79)
L6	0.03	3.03	1.25	0.73	59 (57)	77 (67)
L14	0.12	5.88	2.83	0.54	53 (44)	93 (79)
L15	0.16	7.97	4.58	0.61	43 (44)	94 (72)
L16	0.05	1 22	0.83	0.37	33 (0)	73 (45)
L18	0.10	4.39	1.72	0.56	62 (50)	89 (65)
L19	0.07	1.29	0.92	0.46	30 (48)	68 (70)
L24	0.13	7.49	0.38	0.31	97 (96)	97 (97)
L30	0.24	6.96	2.59	0.51	65 (52)	96 (65)
Group III						
S3	0.02	0.25	0.13	0.38	52 (41)	(−57) (44)
S7	0.02	0 36	0.23	0.12	38 (55)	71 (67)
L11	0.05	1.06	0.45	0.16	60 (59)	89 (72)
L13	0.08	0.54	0.37	0.13	37 (41)	89 (69)
L22	0.04	1.05	0.69	0.61	35 (17)	43 (34)
L23	0.06	1.36	1.08	0.73	21 (13)	48 (9)

E. coli cells prelabelled with ¹⁴C-leucine were irradiated with ultraviolet light and infected with phages as previously described[2] except that the ultraviolet-sensitive bacterial strain was a λpapa lysogen of strain 159 (ref. 12). The ³H-leucine was added 30 min after phage infection and the incorporation was stopped after 10 min. The cells were lysed and proteins were extracted and separated by two-dimensional gel electrophoresis[13]. The ³H/¹⁴C ratio of each r-protein spot, except S1 and L31, was determined. Details of the procedures were described previously[2,10,11]. The data are shown only for the proteins whose synthesis was previously found to be stimulated by λspc1 Synthesis of other proteins was not significantly stimulated by λspc1 compared with the control phage λc1857S7 (λS7). The proteins are classified into groups I and II as described in the text. Group III includes proteins whose synthesis was only weakly stimulated (S3, S7, L13) or whose classification with respect to groups I and II is not clear (L22, L23). The pattern observed with L22 and L23 appears similar to that observed for group I proteins. In the case of L11, the apparent stimulation of its synthesis may be due to contamination of the L11 spot by S5. Concerning a weak reduction by the 116 insertion in the stimulation of the synthesis of group I proteins, we note that stimulation of the synthesis of r-proteins by the transducing phages depends on the multiplicity of infection In the present experiments, the same 'multiplicity' was used, as judged by the absorbance values of the various phage preparations at 260 nm. This corresponds to multiplicity 5 of infectious λS7. Thus, the possible presence of some inactive phage particles in the λspc1–I16 preparation may explain the weak reduction observed in the stimulation of the synthesis of the group I proteins. The slight difference between the two experiments may also be explained on the same basis.
*Similar experiments were done except that the bacterial strain used as a host was a non-lysogenic and ultraviolet-sensitive strain (NO1261)[2]. The values obtained are shown in parentheses.
†Proteins S9 and S11 co-electrophorese in the two-dimensional gel. But, S11 can be made *in vitro* using λspc1 DNA as template but not S9 (ref. 2). Thus, the stimulation of the incorporation of ³H-leucine into this spot is probably a result of the synthesis of S11.

λspc1. Most of these proteins are clearly separated from other proteins in the two-dimensional electrophoresis system used to determine synthesis of r-proteins in the irradiated cells and their synthesis seems to be strongly stimulated by λspc1 infection. Thus this stimulation probably reflects the presence of the structural gene on λspc1.

Physical mapping of insertion mutations

The structure of λspc1, as well as other transducing phages that we have isolated, has been studied by the use of DNA restriction endonucleases (such as EcoRI) and by electron microscopic analysis of various heteroduplexes (Fiandt, Szybalski, Blattner, Lindahl, Jaskunas and Nomura, in preparation). Figure 1 shows the position of various bacterial genes on these transducing phages and the position of the EcoRI sensitive sites on the λspc1 genome.

Both λspc1–I15 and λspc1–I16 had increased phage particle densities (1.489 and 1.491g ml⁻¹, respectively) relative to the parent λspc1 (1.486g ml⁻¹). These increased densities originally suggested that the inactivation of the *spc*ˢ gene on these phages was the result of an insertion. Furthermore, EcoRI digestion of the DNAs from both λspc1–I15 and λspc1–I16 showed a fragment pattern identical to that from λspc1 except that the 17% fragment from λspc1 was replaced by larger fragments in the mutants. These results indicate that the insertions are

in the original 17% fragment which contains the junction of bacterial and λ DNA ("*coli*-λ junction").

The precise position of the insertions as well as the size of the inserted DNAs was determined by heteroduplex analysis. Examples are shown in Fig. 2. In these experiments, the heteroduplexes formed between the mutant (or parent) DNAs and the DNA from λfus2 were examined. λfus2 is a transducing phage carrying the genes *aroE*, *trkA*, *spcA*, *strA*, and *fus*, as well as approximately 28 other r-protein genes (our unpublished experiments, cited in ref. 2). The DNA in λspc1 up to the *coli*-λ junction is homologous to DNA in λfus2 (see Fig. 1). The positions of the insertions obtained from the heteroduplex experiments are indicated in Fig. 1. The I16 insertion was found 270±30 base pairs left of the *coli*-λ junction and the I15 insertion was found 850±40 base pairs left of the junction. Thus, both insertions are in the bacterial DNA. Since the DNA between either insertion and the *coli*-λ junction can code for only one to three r-proteins, we conclude that most or all of the inactivated genes are to the left of the insertions. Independent measurements of the distance between the two insertion sites in the heteroduplex from λspc1–I15 and λspc1–I16 (Fig. 2d) gave the value 610±60 base pairs, which agrees with the calculated value, 580±50.

The sizes of the inserted DNAs were measured and found to be 680±70 base pairs in λspc1–I15 and 1,160±110 base pairs in λspc1–I16. Thus the sizes of these two inserted DNAs
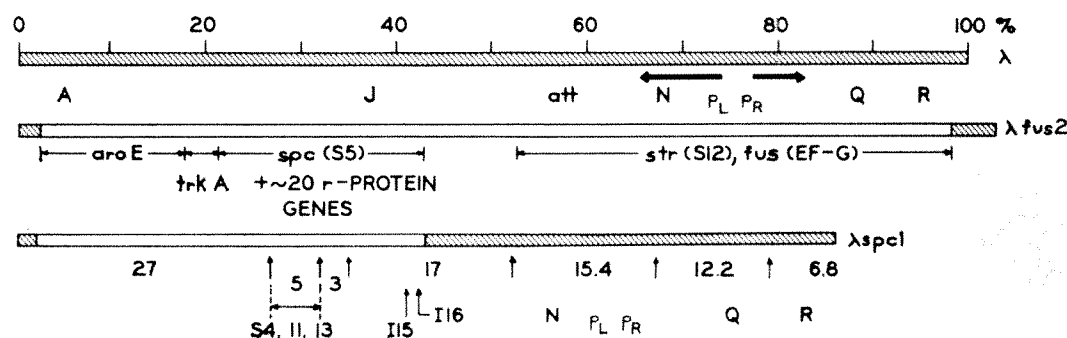
Fig. 1 The structure of λ, λ*fus2* and λ*spc1*. DNA from the λ genome is hatched. Arrows under the λ*spc1* chromosome show the *Eco*RI sensitive sites which produce seven DNA fragments. The size of these fragments is indicated in % λ units. The *str* gene is present in λ*fus2*, but not in λ*spc1*. The structure of λ*fus2* and λ*spc1* is based on the experiments by M. Fiandt, W. Szybalski, F. Blattner, L. Lindahl, R. Jaskunas and M. Nomura (in preparation). Two thick arrows under the λ chromosome indicate the leftwards transcription from the P_L promoter and rightwards transcription from the P_R promoter, respectively. The location of genes for S4, S11 and S13 as well as that of the insertion mutations, I15 and I16, is indicated.

are similar to the sizes of the well-studied insertion DNAs, IS1 (750 to 870 base pairs) and IS2 (1,170 to 1,420 base pairs), respectively[4,5]. The slight difference between our value for I15 and the published values for IS1 is probably not significant.

Direction of r-protein gene transcription at the *str-spc* region

λ*spc1* phage carries about 20 r-protein genes. The data described above show that the insertion of small DNA fragments depresses the expression of many r-protein genes located left of the site of the insertion (compare Fig. 1). The simplest interpretation of the results is that these inactivated genes belong to one transcriptional unit and that the direction of transcription is from right to left in Fig. 1.

Previous experiments suggested that the direction of the transcription of r-protein genes in this region is from *spc* to *str* (ref. 3 and other experiments cited in ref. 1). Since that is opposite to the above expectation, we have reinvestigated the direction of the transcription by determining which strands of λ*spc1* and λ*fus2* hybridise with the r-protein messages for this region.

First, we separated the strands of DNA from λ*trkA*, λ*spc1* and λ*fus2* and determined which strands would hybridise 'leftwards' transcripts and which would hybridise 'rightwards' transcripts. λ*trkA* is a control phage which has only the *aroE-trkA* end of λ*spc1* and λ*fus2*. It does not seem to code

for any r-proteins[2]. For λ*trkA* and λ*spc1*, identification of the polarity of the separated strands was done with purified λ-*l* and λ-*r* transcripts. We found that the 'heavy' strand of λ*trkA* hybridised with the λ-*r* transcript while the 'light' strand of λ*spc1* hybridised with this transcript (Table 2). This method could not be used to determine the polarity of the λ*fus2* strands since they did not hybridise a significant fraction of the standard λ transcripts, presumably because this phage contains only a small fraction (7%) of the λ genome. We therefore used the *in vitro* transcript from the 5% *Eco*RI fragment of λ*spc1* (compare Fig. 1). This transcript ("5% transcript") contained mainly 'leftwards' transcript since it hybridised primarily with the λ*spc1-l* strand (Table 2). λ*fus2* also contains the 5% *Eco*RI fragment and we found that its 'heavy' strand hybridised with the 5% transcript establishing it as the λ*fus2-l* strand (Table 2).

It was previously shown that the *in vivo* synthesised RNA which hybridises to λ*spc1* DNA is almost exclusively r-protein mRNA and its major fraction is homologous to the bacterial DNA where the genes affected by I15 and I16 are located (P. Dennis and M. Nomura, unpublished). Data presented in Fig. 3 show that *in vivo* synthesised mRNA hybridised with the *l*-strands of λ*spc1* and λ*fus2* but not with their *r*-strands. We conclude that *in vivo* transcription of the r-protein genes in the *str-spc* region seems to be exclusively leftwards, that is, in the direction of *str* to *spc*.

Table 2 Hybridisation of *in vitro* transcribed RNA to separated DNA strands of transducing phages

Experiment No.	RNA Type	Input c.p.m.	λ H	λ L	λtrkA H	λtrkA L	λspc1 H	λspc1 L	λfus2 H	λfus2 L
1	λ-r	655	561	23	461	13	44	238	11	49
2	λ-l	560	33	406	27	411	246	49	18	9
3	"5%"	38,983	—	—	104	68	17,631	1,608	10,001	252
4	"λspc1"	27,707	3,439	1,563	3,103	2,355	9,759	3,875	5,510	769

Binding to filters in the absence of DNA [(1) 28 c.p.m.; (2) 22 c.p.m.; (3) 26 c.p.m.; (4) 30 c.p.m.] has been subtracted. Preparation of transcripts from the λ-strands: ^{3}H-labelled transcripts from λ DNA were made in a system with the following composition: 10 mM Tris-HCl (*p*H 7.9), 10 mM MgCl$_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.5 mM K-phosphate, 60 mM KCl, 6% glycerol, 0.6 mM ^{3}H-UTP (1.3 Ci mmol^{-1}), 0.5 mM CTP, 0.5 mM ATP, 0.5 mM GTP, 11 μg ml^{-1} λ DNA and 240 μg ml^{-1} protein of an RNA polymerase preparation, which is approximately 50% pure[14]. The mixture was incubated at 37 °C for 45 min. The reaction was terminated by chilling to 0 °C, 200 μg tRNA was added as carrier and nucleic acids purified by the phenol method. From this preparation 'leftwards' (λ-*l*) and 'rightwards' (λ-*r*) transcripts were purified by preparative hybridisation to λ-L and λ-H strands essentially as described by Bøvre *et al.*[15]. "5% transcript" was prepared in a DNA-dependent protein synthesising system as described previously[14], except that RNA was labelled (with 8.5 Ci mmol^{-1} ^{3}H-UTP) rather than protein. The template used was a 5% fragment of λ*spc1* DNA prepared by *Eco*RI digestion (see Fig. 1). Its molecular weight is about 1.4×10^6 and it codes for the r-proteins S4, S11 and S13 (Lindahl, Zengel and Nomura, in preparation). λ*spc1* transcript was prepared using λ*spc1* DNA as a template according to the method used for the λ transcripts (see above). Separation of strands was performed by annealing with poly(U,G) and isopycnic CsCl centrifugation as previously described[15,18]. Liquid hybridisation was performed in 2 × SSC using a large excess of DNA. Samples were incubated at 67 °C for 4–6 h and collected on Millipore HAWP nitrocellulose filters. Each filter was washed with 50 ml 2 × SSC. The filters were then incubated in 20 μg pancreatic and 2.5 units T1 ribonucleases ml^{-1} in 2 × SSC (1.5 ml per filter) for 1 h at 37 °C. Finally the filters were washed twice in 2 × SSC (10 ml per filter), dried, and the radioactivity bound to each filter was measured. SSC contains 0.15 M NaCl, 0.015 M Na citrate, *p*H 7.2.

*DNA strand H and L designate separated strands which were located at heavier and lighter density positions, respectively, in the isopycnic CsCl centrifugation after annealing with poly(U,G).
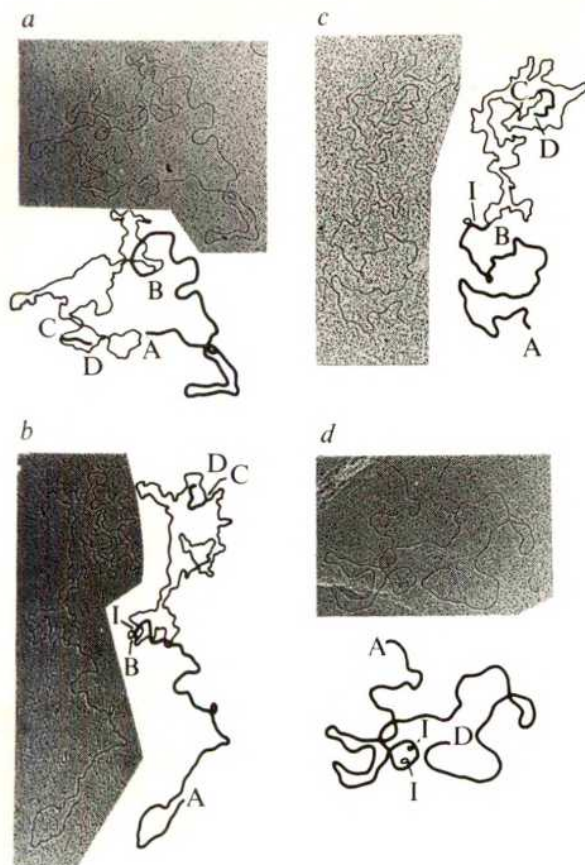
Fig. 2 Electron micrographs of heteroduplex DNA molecules. *a*, λ*spc*1 and λ*fus*2 DNA; *b*, λ*spc*1–I16 and λ*fus*2 DNA; *c*, λ*spc*1–I15 and λ*fus*2 DNA; *d*, λ*spc*1–I16 and λ*spc*1–I15 DNA. Both phage PM2 DNA (9,500 base pairs; refs 19 and 20 and W. Szybalski, personal communication) and ΦX174 DNA (5,250 base pairs; ref. 20) were added as references for length measurements. In *a* and *b*, one PM2 DNA molecule overlaps on the heteroduplex molecules. The tracings of each heteroduplex are shown together with the electron micrographs. Heavy lines are duplex regions, and light lines are single-strand regions. Several key positions are indicated as follows: A, the left end of the heteroduplex molecule (compare Fig. 1); B, the position of the right *coli*-λ junction in λ*spc*1; C, the position of the right *coli*-λ junction in λ*fus*2; D, the right end of the heteroduplex molecule; I, the position of insertion (I15 or I16). The method for preparation of heteroduplexes and for electron microscopy was according to ref. 16. The lengths of double strand DNA regions were measured using PM2 DNA as a reference. The lengths of single strand DNA regions were measured using ΦX174 DNA as a reference, but in some experiments, PM2 DNA was used as a reference. No significant difference was observed between the values obtained with the two methods.

Both λ*spc*1 DNA and its 5% *Eco*RI fragment code for r-proteins in our DNA-dependent protein synthesising system. We found that the *in vitro* transcripts from these DNAs also hybridised predominantly to the *l*-strand of λ*spc*1 DNA (Table 2). Since the 5% fragment seems to contain only r-protein genes (L. L., J. Zengel and M. N., unpublished), we conclude that the *in vitro* transcripts from this fragment contain predominantly r-protein messengers. *In vitro* transcripts of λ*spc*1 seem to contain transcripts from the λ genes in addition to the transcripts from the bacterial genes, as can be seen from hybridisation to the separated λ DNA. To test the transcripts from the bacterial DNA, we performed hybridisation experiments using separated λ*fus*2 DNA strands which contain only a small fraction of the λ genome. The results showed that the *in vitro* transcripts for λ*spc*1 hybridised mainly with the λ*fus*2-*l* strand (Table 2, experiment 4). Thus, these results are consistent with the finding that *in vivo* synthesised r-protein mRNA for the genes on λ*spc*1 are transcribed leftwards and

indicate that in general the correct strand is being transcribed under our conditions for *in vitro* transcription.

Multiple transcriptional units

We have shown that insertion mutations, I15 and I16, greatly reduce the expression of many r-protein genes present on the λ*spc*1 genome. A few of the r-protein genes on this phage, however, are apparently not affected to any large extent by these insertions. Among them, the genes for S4, S11, and S13 have been shown to be closely linked to each other and are mapped on the 5% *Eco*RI fragment (Fig. 1 and L. L., J. Zengel and M. N., unpublished). Most of the genes inactivated by the I15 or I16 insertions (genes for the group II proteins in Table 1) have been mapped between the gene cluster for S4, S11 and S13 and the *coli*-λ junction using several deletion mutants of transducing phages (our unpublished experiments). Because the direction of the transcription is from right to left, as demonstrated in our present work, the inactivated genes are distal to the insertion. Although the mechanism of this inactivation of the distal genes by these insertions is not known, the size of the inserted DNA as well as their "polar effect" resemble those of the IS1 and IS2 insertions studied in other systems[4–6]. It is very likely that the r-protein genes affected by the I16 (or I15) insertion belong to a single transcriptional unit, and those not affected (S4, S11 and S13, and others) belong to another one or more transcriptional units. Our present data cannot, however, exclude the possibility that there
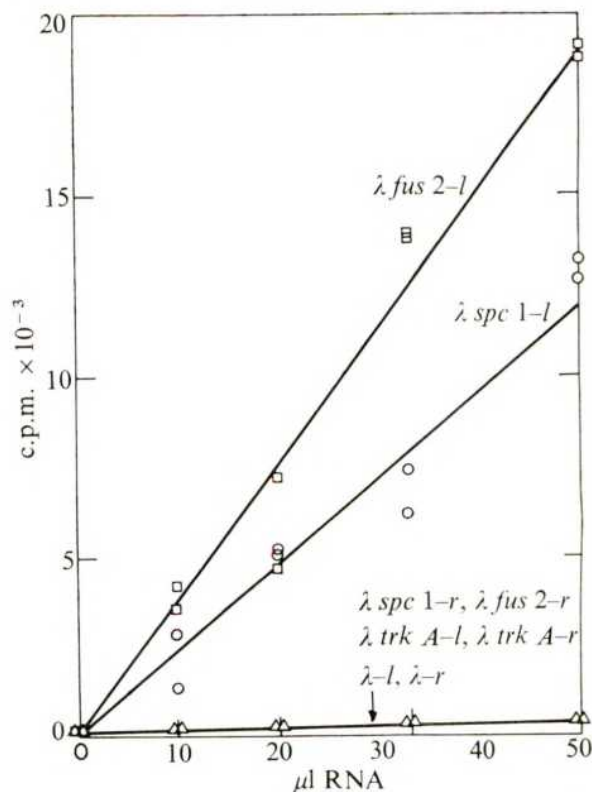
Fig. 3 Hybridisation of *in vivo* RNA to separated strands of λ, λ*trkA*, λ*spc*1, and λ*fus*2. Various amounts of RNA prepared from pulse labelled *E. coli* K12 (see below) were hybridised to DNA as indicated. All strands were used in an excess over RNA. Triangles show the average of the bindings to the indicated strands. Binding to individual strands differs slightly, but not enough to be shown on this scale. Preparation of RNA: *E. coli* K12 PR13 (ref. 17) was grown exponentially in glucose–amino acid medium at 37 °C[11]. At approximately 10^8 cells ml^{-1} the culture was labelled with 0.5 μg ³H-uracil (25 Ci mmol^{-1}) ml^{-1} for 4 min. The pulse was terminated by pouring the culture on crushed ice. RNA was prepared from the cells essentially as described before[11]. The RNA preparation had 1.2×10^7 c.p.m. ml^{-1}. Conditions for hybridisation are described in the legend to Table 2.

is a "secondary" promoter between the genes affected and those not affected and that all the r-protein genes carried by λspc1 are cotranscribed in "normal" conditions. The activity of such a secondary promoter could be enhanced by an upstream polar mutation. The present data also cannot exclude the possibility that the genes inactivated by a single insertion belong to more than one transcriptional unit and the inactivation involves a more complicated mechanism. Thus, further studies are required to elucidate the mechanism of the apparent polar effect observed in the present system.

It should also be noted here that while the polar effects resulting from IS1 insertions are very strong in other systems, those resulting from the I15 insertion in our system are only partial. The question whether the IS1 DNA is identical to the DNA inserted in λspc1-I15 must await further experiments.

The genes on λspc1 that code for the group II proteins (Table 1) can function *in vivo* in the presence of the λ repressor (experiments in Table 1 and unpublished data) and so the transcription probably starts from a bacterial promoter, rather than a λ promoter. Such considerations would suggest the presence of a bacterial promoter close to the *coli*-λ junction in λspc1. No experimental proof has been obtained to support this conjecture, however.

Previous experiments using phage Mu-1 suggested that the r-protein genes in the str–spc region are transcribed in the direction of spc to str (ref. 3 and other experiments cited in ref. 1). Our present results demonstrate that the opposite direction is correct. We cannot offer any simple explanation for the results of the Mu insertion experiments. In the previous experiments, the genes inactivated by the Mu phage insertion were not recovered and thus the possibility that these genes were deleted rather than inactivated by a polar effect was not eliminated.

Previous physiological studies on the synthesis of r-proteins in *E. coli* suggested the presence of several transcriptional units for r-protein genes[7]. It is now clear that the organisation of r-protein genes is complex. Many r-protein genes (about 30) are clustered at the str–spc region. Our results strongly suggest that they are organised into at least two transcriptional units. Furthermore, we have previously shown that at least one r-protein gene, the structural gene for S18, is mapped outside

the str–spc region[8,9]. We, and others, have also observed that approximately five other r-protein genes are clustered near *rif* which is far away from the str–spc region (S. R. J., L. L. and M. N., unpublished and J. Friesen, personal communication). Thus, coordinated synthesis of all the r-proteins[10,11] requires a mechanism which coordinates the expression of several r-protein gene transcriptional units.

1 Jaskunas, S. R., Nomura, M., and Davies, J., in *Ribosomes* (edit. by Nomura, M., Tissières, A., and Lengyel, P.), 333–368 (Cold Spring Harbor Laboratory, New York, 1974).
2 Jaskunas, S. R., Lindahl, L., and Nomura, M., *Proc. natn. Acad. Sci. U.S.A.*, 72, 6–10 (1975).
3 Nomura, M., and Engbaek, F., *Proc. natn. Acad. Sci. U.S.A.*, 69, 1526–1530 (1972).
4 Hirsch, H. J., Starlinger, P., and Brachet, P., *Molec. gen. Genet.*, 119, 191–206 (1972).
5 Malamy, M. H., Fiandt, M., and Szybalski, W., *Molec. gen. Genet.*, 119, 207–222 (1972).
6 Saedler, H., Reif, H. J., Hu, S., and Davidson, N., *Molec. gen. Genet.*, 132, 265–289 (1974).
7 Molin, S., von Meyenburg, K., Gulløv, K., and Maaløe, O., *Molec. gen. Genet.*, 129, 11–26 (1974).
8 Bollen, A., *et al.*, *J. molec. Biol.*, 76, 463–472 (1973).
9 Kahan, L., Zengel, J., Nomura, M., Bollen, A., and Herzog, A., *J. molec. Biol.*, 76, 473–483 (1973).
10 Dennis, P., *J. molec. Biol.*, 88, 25–41 (1974).
11 Dennis, P., and Nomura, M., *Proc. natn. Acad. Sci. U.S.A.*, 71, 3819–3823 (1974).
12 Murialdo, H., and Siminovitch, L., in *The Bacteriophage Lambda* (edit. by Hershey, A. D.), 711–723 (Cold Spring Harbor Laboratory, New York, 1971).
13 Kaltschmidt, E., and Wittmann, H. G., *Analyt. Biochem.*, 36, 401–412 (1970).
14 Kaltschmidt, E., Kahan, L., and Nomura, M., *Proc. natn. Acad. Sci. U.S.A.*, 71, 446–450 (1974).
15 Bøvre, K., Lozeron, H. A., and Szybalski, W., in *Methods in Virology* (edit. by Maramorosch, K., and Koprowski, H.), 5, 271–292 (Academic, New York, 1971).
16 Davis, R. W., Simon, M., and Davidson, N., *Methods in Enzymology* (edit. by Grossman, L., and Moldave, K.), XXI, 413–428 (Academic, New York, 1971).
17 Reiner, A. M., *J. Bact.*, 97, 1437–1443 (1969).
18 Avitabile, A., *et al.*, *Molec. gen. Genet.*, 132, 1–12 (1974).
19 Espejo, R. T., Canelo, E. S., and Sinsheimer, R. L., *Proc. natn. Acad. Sci. U.S.A.*, 63, 1164–1168 (1969).
20 Sharp, P. A., Hsu, M. T., Ohtsubo, E., and Davidson, N., *J. molec. Biol.*, 71, 471–497 (1972).
21 Sparling, P. F., Modolell, J., Takeda, Y., and Davis, B. D., *J. molec. Biol.*, 37, 407–421 (1968).

Simplest statistical geometric model of the simplest version of the multicomponent random packing problem

J. A. Dodds

Laboratoire des Sciences du Génie Chimique, 54000 Nancy, France

A simple, statistical, geometric model of multicomponent random disk packing, which ignores the gaps that occur in real packings, is compared with experimental packings. The model gives a good qualitative description of the changes that occur in packing structures as the proportions of the disks in a mixture changes, and considers the different types of contact that occur between disks. The average coordinance for disk packings is constant for all mixture proportions.

THE sphere packing problem, common to many fields of science and technology, arises in studies of systems involving bubbles, drops, grains, fibres, molecules, and so on[1].

The packing of monosize components is, however, considered

most often and then from the point of view of overall or average properties (such as porosity). That is in spite of the fact that, in practice, mixtures of different sized components are nearly always encountered; in cases such as these the distribution effects (for example pore-size distribution) are very important. In such cases, however, the calculation of the fine structure of the packing is impossible and even measurement is difficult. We therefore present here an alternative approach.

The two-dimensional packing of a mixture of two disk sizes, probably the simplest possible expression of the sphere packing problem, can be considered by using a model designed for multicomponent sphere packing[2,3] but reduced here from three to two dimensions.

The basic hypothesis is that in a random arrangement of disks each disk touches its neighbours. This allows the space to be divided up into triangles formed by lines joining the

centres of contiguous disks (Fig. 1). The hypothesis is, therefore, that gaps, such as those shown by the dotted lines in Fig. 1, do not exist.

This abstraction of the problem allows the effects of size distribution to be considered apart from the less easily defined effects of the perturbation of the structure caused by inevitable,
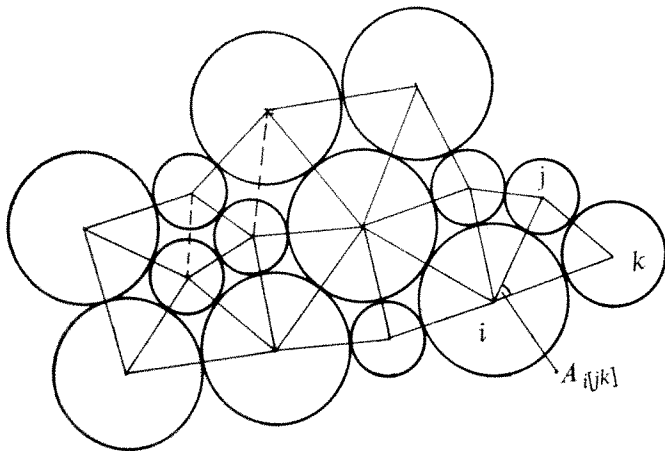
Fig. 1 Binary disk packing.

irregular gaps. That is, elementary subunits in the packing are calculated but their subsequent assembly to form the packing structure is ignored. The model is limited to the case in which there is interaction between the sizes; effectively to cases in which the diameter ratio is less than 1:6.46. Above that value it is possible to fit a small disk in the space between three large contiguous disks without touching them.

Mathematical statement of the model

In the case of the binary packing of disks of sizes 1 and 2 the space is divided into four types of triangular subunits, each formed by groups of three disks—111, 112, 122, 222. More generally, in an N component disk packing there will be

$$N(N+1)(N+2)/3! \tag{1}$$

different types of triangle. Once the frequency distribution of these different triangles is known, then all of the properties of the idealised packing can be deduced: the porosity, the pore size distribution, the mean coordination number for each disk size, and so on.

Fig. 2 Triangle frequency distribution. Type 111: ×, experimental distribution; a, model distribution. Type 112: ●, experimental distribution; b, model distribution. Type 122: +, experimental distribution; c, model distribution. Type 222: ○, experimental distribution; d, model distribution.
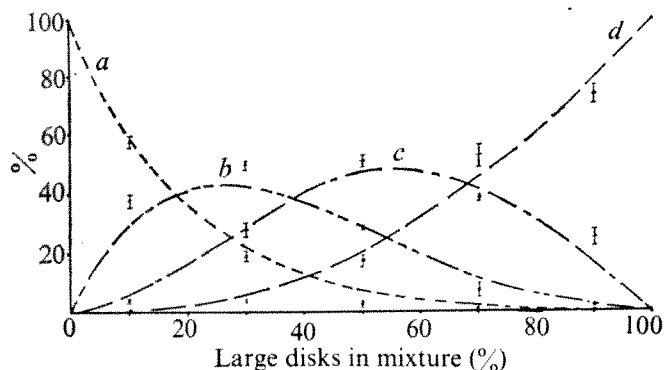
The triangle frequency distribution must be calculated from the relative numbers and sizes of the disks. Two effects come into play: size and number. For example, if there are more disks of diameter 1, then triangles including disks of diameter 1 will be favoured. On the other hand, disks of diameter 2, being bigger, will have more other disks touching them and will form more triangles, therefore, triangles including disks of diameter 2 will be favoured. The probability of a disk taking part in a triangle will be related to Z_i:

$$Z_i = n_i C_i$$

where n_i is the fractional number frequency of disks of size i in the packing, and C_i is the average number of contacts on disks of size i in the packing.

If triangle systems are obtained by random combinations of groups of three disks, then the frequency distribution of triangles in a binary mixture will be given by the respective terms of the expansion of the expression

$$(Z_1+Z_2)^3 \tag{2}$$

or, in a form suitable for programming,

$$(3!/a!\,b!)(Z_1)^a(Z_2)^b \tag{3}$$

where a or $b = 0$, 1, 2 or 3 and $a+b = 3$. That is, the relative frequency of triangles of type 112 is given by

$$(3!/2!\,1!)(Z_1)^2(Z_2)^1 \tag{3}$$

The mean coordinate number for a given disk size is the reciprocal of the mean angle subtended at that type of disk by other disks in the packing, expressed in fractions of a circle. This is not known *a priori* and must be calculated by trial and error. Equations (1)–(3) show that:

$$C_j = (Z_i+Z_j+Z_k+ \ldots)^2/A' \tag{4}$$

where
$$A' = (A_{j(ii)} Z_i{}^2 + A_{j(jj)} Z_j{}^2 + A_{j(kk)} Z_k{}^2 + \ldots)$$
$$+ 2 (A_{j(ij)} Z_i Z_j + A_{j(ik)} Z_i Z_k + A_{j(jk)} Z_j Z_k + \ldots)$$

$A_{i(jk)}$ (Fig. 1) is the angle subtended at the centre of disk i by the lines joining its centre with the centres of two contiguous disks, j and k. For diameter ratios up to the maximum possible considered here (1:6.46), to within about 1%:

$$(A_{j(ii)})^{\frac{1}{2}} \times (A_{j(jj)})^{\frac{1}{2}} \approx A_{j(ji)} \tag{5}$$

This allows equation (4) to be simplified to

$$C_j = \left[\sum_{i=1}^{i=N} Z_i \Big/ \sum_{i=1}^{i=N} K_{ji} Z_i \right]^2 \tag{6}$$

where $K_{ji} = (1/C_{ji})^{\frac{1}{2}}$ and C_{ji} is the number of contacts on a disk type j surrounded completely by disks type i. Equations like (4) or (6) can be solved rapidly and efficiently using the Newton–Raphson technique.

In all calculations made with ternary as well as binary mixtures, with diameter ratios up to the maximum allowed by the model:

$$\sum_{i=1}^{i=N} n_i C_i = \text{constant} \tag{7}$$

For disks, this constant is 6.0.

No formal proof of this has been obtained but the reproducibility of the value 6.0 suggests something more than a

purely fortuitous relationship of the type seen in equation (5). This is perhaps connected with the proven fact that the average Voronoi polygon of any array of points in two dimensions has exactly six sides[4].

Experimental binary disk packings

Experiments have been performed with large disks (20 mm and 40 mm diameter) to be able to distinguish between real contacts and close contacts, based on differences of about 1% of a diameter. Disks were made from steel washers machined to be perfectly circular with a tolerance on the diameter of ± 0.2 mm. To form a two-dimensional packing they were introduced into a

in Fig. 1. The numbers of the different types of 'false' triangle were then counted (Table 1 and Figs 2 and 3).

Figure 2 is the sum of the distributions of the 'true' and the 'false' triangles, as it was found that these two distributions did not differ greatly from each other.

The model

From the results in Figs 2 and 3 and the ratios C_4/C_2 (Table 1), it can be seen that the model gives a good qualitative picture of the variations in packing characteristics with mixture proportions. Table 1 shows also that the total number of contacts on a given number of disks is approximately constant,

Table 1 Model and experimental results

| Disk diameter | % | Number of triangles per experiment | | | Model | | | Coordination numbers | | Experiment | |
		True	False	C	C_4/C_2	Σ_{nc}	C	σ	C_4/C_2	Σ_{nc}
2	90	274	396	5.71			4.39	0.74		
4	10			8.64	1.51	6.00	6.89	0.77	1.39	4.64
2	70	197	188	5.25			4.18	0.64		
4	30			7.74	1.47	6.00	6.49	0.88	1.56	4.87
2	50	148	196	4.91			3.82	0.63		
4	50			7.09	1.35	6.00	5.68	0.78	1.49	4.75
2	30	96	212	4.63			3.56	0.51		
4	70			6.59	1.42	6.00	5.10	0.74	1.44	4.64
2	10	82	121	4.41			3.15	0.64		
4	90			6.18	1.40	6.00	4.88	0.75	1.54	4.71

360 by 600 mm Perspex frame one by one in groups of ten, with the required mixture proportions. The packing was not shaken, so as to avoid segregation effects which can be important in mixtures. Each packing was repeated at least twice and represents a counting of around 500 disks, depending on size.
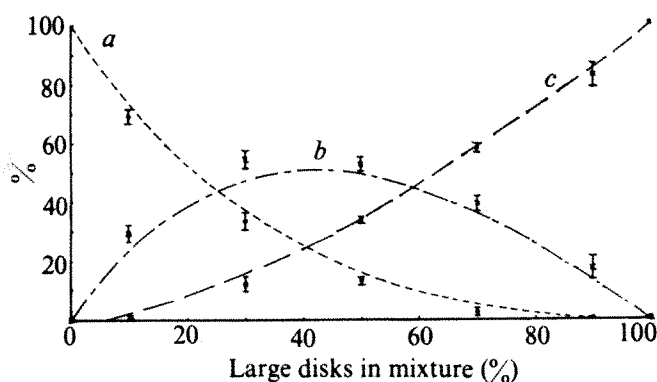
Fig. 3 Types of contact. Type 11: ●, experimental; *a*, model. Type 12: ×, experimental; *b*, model. Type 22: ■, experimental; *c*, model.

Three different mixtures were used. A tracing of the assembly was made and the various countings were made on diazo prints of this tracing. In all cases, disks at the edges to a depth of about two disks were excluded to minimise edge effects.

The centres of contiguous disks were joined and several different counts were made: the numbers of the different types of 'true' triangle; the numbers of contacts on each disk size; the numbers of the different types of contact—that is, between similar disks—1,1 and 2,2—and between dissimilar disks—1,2. The remaining polygons were divided into triangles by joining the closest non-touching disks, as by the dotted lines

as is predicted by the model.

The model describes high density packing with a maximum number of contacts between disks. Not all of these contacts are made, however; it seems that the selection of the contacts not to be made is done randomly and that the gaps do not tend to be associated preferentially with either of the two disk sizes. The influence of the gaps is found only in the absolute values: for example, the total number of contacts per unit sample of disks is 4.72 and not 6. The fact that this reduction is uniform for all mixtures could be used to make the model quantitatively predictive.

It may be concluded that the triangle frequency distribution and the distribution of contact types are not much disturbed by the presence of the gaps. This indicates that, contrary to the influence of the walls, the gaps do not have any ordering influence and that, as the model assumes, the disks touch each other on the basis of random encounters governed only by their relative size and number.

The results also provide some insight to another feature of binary packings, which has been noticed before. Since each type of triangular unit has its own characteristic porosity the variation of the overall porosity with mixture proportions is given by a weighted sum of the four distribution curves in Fig. 2. This provides an explanation for the occurrence of the well known V-shaped, porosity/mixture proportion curves for binary packings. This effect is much more noticeable for sphere packings or disk packings with a wider diameter ratio.

Several points emerge from this study: packings are formed by random encounters between disks; the model predicts the correct distribution of contact types and triangular subunit types; the total number of contacts in a unit sample of disks is constant for all mixture proportions; the gaps in the packing occur in a random fashion.

1 Haughey, D. P., and Beveridge, *Can. J. chem. Engng*, 47, 130–140 (1969).
2 Wise, M. E., *Philips Res. Rep.*, 7, 321–343 (1952).
3 Hogendjik, M. J., *Philips Res. Rep.*, 18, 109–126 (1963).
4 Smith, C. S., *Metal Interfaces*, 65 (American Metals Society, Cleveland, 1965).

letters to nature

Discovery of a corona around an early-type star and the problem of mass loss

THE star τ Sco, HD149438, spectral type B0 V, was observed spectroscopically by the Princeton telescope-spectrometer on the Copernicus satellite during June and July 1973. This star is well known[1] as being one of the few early type stars with a small projected rotational velocity, v-sin$i$ = 20 km s^{-1}, thus showing narrow spectral lines with a minimum of overlap or blending. So the spectrum of the star was scanned with a resolution of 0.05 Å over the entire wavelength range from 950 Å to 1,560 Å to obtain a prototype ultraviolet spectrum for an early-B star. This spectrum is in preparation by Rogerson and Upson.

Apart from the numerous spectral features with a full width at half maximum (FWHM) of about 20 km s^{-1} which originate in the photosphere of the star, and the interstellar absorption lines, the spectrum also shows a few very wide absorption lines. These lines are identified as belonging to the spectra of O VI at 1,032 and 1,038 Å, N V at 1,239 and 1,243 Å and Si IV at 1,394 and 1,403 Å. The lines are highly asymmetric with the largest depression close to the laboratory wavelengths and an enhanced short-wavelength wing, which extends to a Doppler velocity of about $-1,000$ km s^{-1}. This suggests that the lines are formed in the expanding outer layers of the atmosphere.

The strong presence of O^{5+} and N^{4+} ions in the outer layers of the star is surprising. From the visual and ultraviolet spectrum the temperature of the stellar photosphere is determined to be about 30,000 K and the stellar atmosphere models predict that the temperature in the layers above the photosphere is less. To produce a significant fraction of the observed highly ionised atoms, the temperature in the outer layers must be[2] of the order of 1×10^5–5×10^5 K. (The production of O^{5+} and N^{4+} by radiative ionisations can be neglected since the required stellar radiation in the far ultraviolet is very strongly absorbed in the photosphere by neutral hydrogen atoms and ionised helium atoms.) Therefore, the temperature in the atmosphere of τ Sco must decrease with height in the photosphere, go through a minimum in higher layers and then increase again to at least a few hundred thousand K.

A similar temperature structure is known in the solar atmosphere, where the minimum is about 4,200 K and from there on the temperature increases outward throughout the chromosphere until it reaches a more or less constant value of about 2×10^6 K in the corona. The large temperature is due to the dissipation of mechanical energy in the form of acoustic waves in the lower chromosphere and possibly by Alfven waves in the corona[3]. The mechanical energy in the Sun is supposed to originate in the unstable hydrogen convection zone below the photosphere.

Main sequence stars with effective temperatures greater than about 8,300 K are not expected to have well developed hydrogen convection zones nor to generate a large mechanical energy flux which could produce a stellar corona[4].

A possible mechanism for generating a mechanical energy flux in hot stars has been proposed by Hearn[5,6]. He has pointed out that in the presence of a strong radiation field,

such as in early type stars, density waves can grow and propagate outwards due to the absorption of radiation. This theory predicts a mechanical energy flux of the order of 10^7 erg cm^{-2} s^{-1} for a main sequence B0 star, which in analogy to the solar chromosphere, seems to be sufficient to heat the outer layers to a few hundred thousand K.

The presence of a high temperature region (called a corona, in analogy to the solar case) around early type stars may provide the clue to the understanding of mass loss from hot stars. The observed expansion velocities are generally accepted to be caused by radiation pressure acting primarily on the resonance lines of abundant ions[7]. An important limitation to the efficiency of this mechanism is the fact that the line transitions which are most suitable for absorbing momentum from the radiative flux in the outer atmospheric layers are also extremely effective in the photospheric layers in the reduction of the needed flux. This problem can be illustrated by the very small rates of mass loss predicted by Lucy and Solomon for stars with effective temperatures slightly greater than 30,000 K, at type B0, where the photospheric C IV lines are very strong.

This difficulty is overcome if the outer atmosphere is hotter than the photosphere, because the abundant ions in the outer atmosphere are different from the photospheric constituents. The presence of O VI ions in the corona, having resonance lines at wavelengths near the stellar flux maximum, is especially important in this respect. If 10% of the coronal oxygen atoms are in the form of O^{5+} and the oxygen abundance is the same as in the Sun, the outward directed radiative force exerted on the gas due to the O VI lines exceeds the inward directed gravitational attraction by a factor of four in τ Sco. A preliminary estimate indicates a rate of mass loss of about $10^{-8} M_\odot$ yr^{-1}. This is about two orders of magnitude less than the mass loss rate from the Orion OB supergiants[8].

How unique is τ Sco? In its visible spectrum there is no indication that the star is different from other B0 V stars, except for its small projected rotational velocity. If the star is normal, as we expect, other early-B stars may be surrounded by coronae as well. A preliminary check of the Copernicus files has indicated that traces of O VI lines are detectable on the lower resolution spectra of several other early type stars. Further details will be published elsewhere.

J. B. ROGERSON, JR
H. J. G. L. M. LAMERS*

Princeton University Observatory,
Peyton Hall, Princeton, New Jersey 08540

1 Uesugi, A., and Fukuda, I., *Contrib. Inst. Astrophys. and Kwasan Obs.*, No. 189 (University of Kyoto, 1970).
2 Jordan, C., *Mon. Not., R. astr. Soc.*, 142, 501–521 (1969).
3 Jordan, S. D., in *Stellar Chromospheres* (edit. by Jordan, S. D., and Avrett, E. H.), SP-317, 181–201 (NASA, 1973).
4 De Loore, C., *Astrophys. Space Sci.*, 6, 60–100 (1970).
5 Hearn, A. G., *Astr. Astrophys.*, 19, 417–426 (1972).
6 Hearn, A. G., *Astr. Astrophys.*, 23, 97–103 (1973).
7 Lucy, L. B., and Solomon, P. M., *Astrophys. J.*, 159, 879–893 (1970).
8 Morton, D. C., *Astrophys. J.*, 150, 535–542 (1967).

North–south component of the interplanetary magnetic field and large scale auroral dynamics

THE role of the north–south component B_z of the interplanetary magnetic field on magnetospheric processes, in particular on magnetospheric substorm processes, has caused some controversy[1-3]. It has been generally thought that the southward turning of the B_z component leads directly to a series of growth phase processes which trigger the expansive phase of substorms. On the other hand, auroral observations made along the meridian chain of stations in Alaska showed that substorms occur frequently along a contracted auroral oval (namely, beyond the poleward field of view at auroral zone stations) even when the B_z component is directed northward $(B_z > 0)$[4].

Fortunately, both large scale auroral photographs taken from the DMSP satellite (October and December 1972, and January 1973), and the interplanetary magnetic field data during the corresponding periods (from the HEOS satellite) have recently become available. The results are ideal for

frequency of substorms for contracted ovals[5]. Thus, on the basis of the ISIS-2 and DMSP–HEOS satellite data, one can conclude that the occurrence of substorms does not depend on the size of the oval, except when the oval becomes minimum (that is, during prolonged periods of a large positive B_z component).

I now examine theoretical implications of this finding. Let φ_D be the production rate of merged (or open) field lines along the dayside X line. This quantity is also believed to be equal to the potential drop along the X line and also to the potential drop across the dawn–dusk meridian in the polar cap[6,7]. φ_N is the production rate of reconnected (or closed) field lines along the nightside X line.

Consider the quantity defined by

$$S = \int_0^\tau (\varphi_D - \varphi_N)\,dt$$

where the time $t = 0$ is chosen at the time when the interplanetary magnetic field begins to decrease after having a large northward component for an extended period. Note that the quantity S defined here is equal to $(A_1 - A_0)B_p$, where A_0 and A_1 denote the area of the minimum size oval and of an expanded oval, and B_p denotes the magnetic field intensity
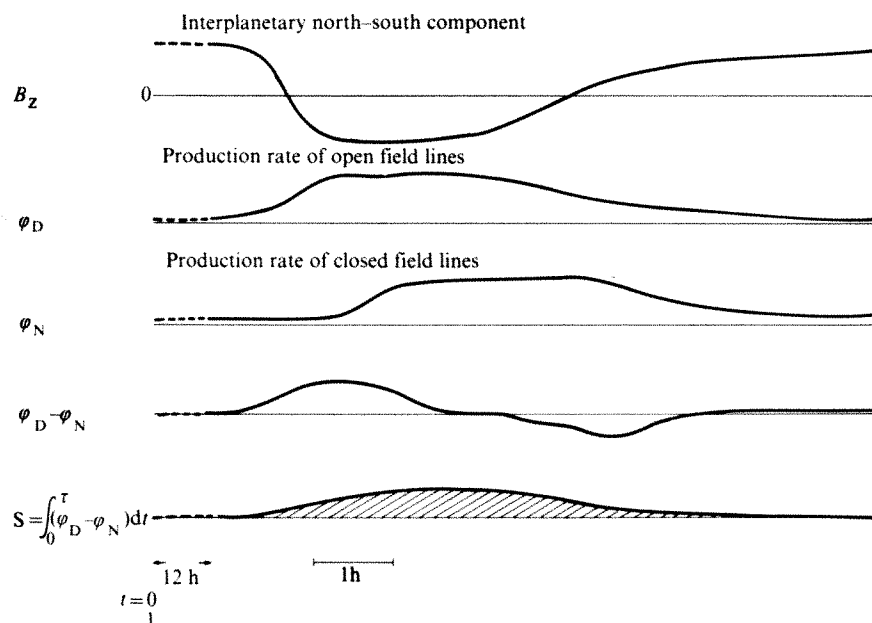
Fig. 1 Hypothetical change of the B_z component and the resulting changes of φ_D, φ_N, $\varphi_D - \varphi_N$ and

$$S = \int_0^\tau (\varphi_D - \varphi_N)\,dt.$$

settling the controversy; it seems that the occurrence of substorms does not depend on the size of the auroral oval, except for periods when the oval contracts to its minimum size (midnight latitude $\simeq 72°$).

Full details of this study will be reported in detail elsewhere. In brief, the occurrence of auroral substorms depends on neither the sign of the B_z component, nor the sign of the dB_z/dt, except for prolonged periods ($\gtrsim 6$ h) of the large ($\gtrsim 5\gamma$), steady, positive (northward) B_z field. During these exceptional periods, the midnight portion of the oval contracts to invariant latitude of about $72° \sim 73°$ (the highest latitude observed so far for a quiet oval). Thus, this contracted oval can be called the minimum size oval. No substorm features are observed along the minimum size oval; the corresponding state of the magnetosphere may be considered to be the 'ground state'.

What magnetospheric quantities can one infer, on the basis of these findings, as a controlling factor of the occurrence of substorms? Photographs taken from ISIS-2 have already indicated that there is no drastic decrease of the occurrence

in the polar ionosphere. Thus, we may write

$$S = \int_0^\tau (\varphi_D - \varphi_N)\,dt = B_p(A_1 - A_0)$$

Figure 1 illustrates schematically time variations of φ_D, φ_N, $(\varphi_D - \varphi_N)$ and S when the north–south component of the interplanetary magnetic field B_z varies from a large positive value to a negative value for about two hours and then back to a large positive value again. Note that an extended period of a large positive B_z value is the initial condition.

The immediate response of φ_D to the B_z change has been observed[6]; the motion of the cusp or of the mid-day aurora towards the Equator closely follows B_z changes. But it is not yet known accurately how rapidly the midnight portion of the oval responds to the initial B_z change. This depends on how quickly the information on a change of φ_D can reach the nightside X line and the production rate φ_N begins to

respond to it. The minimum delay time τ_m can be roughly estimated by

$$\tau_m = B_p(A_1 - A_0)/\varphi_D \simeq 40 \text{ min}$$

where

$$\varphi_D = (400 \text{ km s}^{-1}) \times (5\gamma) \times (15 R_E)$$

and A_0 and A_1 are assumed to be bounded by the latitude circles of 72° and 65°, respectively.

Because of this delay, the quantity $(\varphi_D - \varphi_N)$ should increase initially. This quantity is directly proportional to the rate of change of the polar cap area, namely the area bounded by the auroral oval. When $(\varphi_D - \varphi_N)$ is positive, the oval expands. But φ_N begins to increase about 40 min to 1 h after $t = 0$, and eventually a new steady state $\varphi_D = \varphi_N$ will be reached. Then the auroral oval ceases to expand.

Suppose the B_z then begins to increase after maintaining a large negative value for about two hours. The quantity φ_D responds immediately to the B_z change and begins to decrease. But the corresponding φ_N variation will again be delayed for about 40 min to 1 h, until the new information on B_z can reach the nightside X line. During this period, the quantity $(\varphi_D - \varphi_N)$ becomes negative, and thus the oval contracts. If a large B_z value is maintained for a prolonged period, both φ_D and φ_N reach the same minimum value.

Meanwhile, the quantity S increases until $(\varphi_D - \varphi_N)$ becomes zero, and then begins to decrease. Eventually, S will become zero after a prolonged period of a large B_z value (that is, when both φ_D and φ_N reach the same minimum value).

In constructing Fig. 1, I assumed that both φ_D and φ_N have a finite value even when the B_z component has a large positive value. This is because the magnetotail (a product of the solar wind–magnetosphere dynamo) seems to be a permanent feature. At the lunar distance $(60 R_E)$, there is no significant difference in the structure of the magnetotail for both low K_p values $(K_p \leqslant 1+)$ and high K_p values $(K_p \geqslant 2-)$[10]. Thus, there must always be a finite amount of open flux $(= B_p A_0)$,
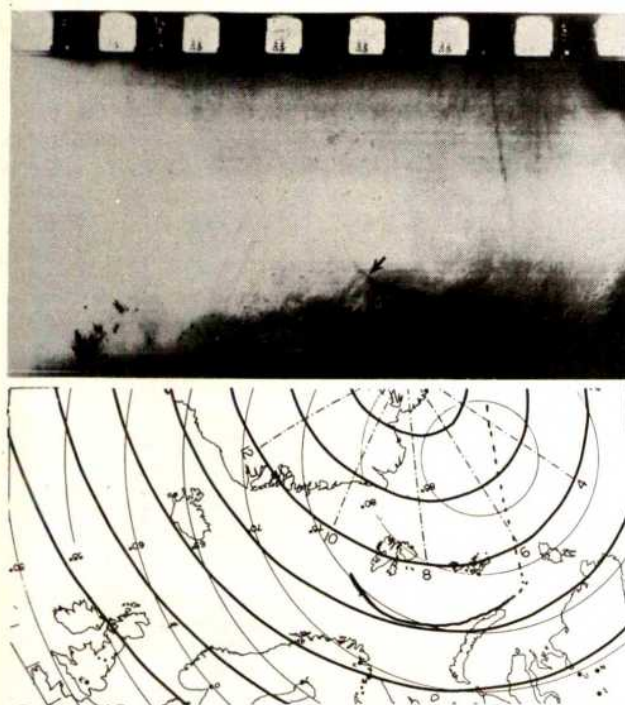
Fig. 2 DMSP photograph (in negative) taken at 2057 UT on December 24, 1972, together with an approximate geographic area covered by the photograph; it shows also the invariant latitude lines at 100 km altitude (thick lines) and geographic latitude lines (thin lines).

Fig. 3 DMSP photograph (in positive) taken at 1252 UT on January 12, 1973; see the caption for Fig. 2.

even when B_z has a large positive value for an extended period. During such a period, the auroral oval has the minimum area A_0.

So the magnetospheric substorm can be considered as a process by which the magnetosphere tends to remove the excess energy ε_s associated with S. When the auroral oval at a particular time is significantly greater than its minimum size,

$$\varepsilon_s \simeq (L/R_T^2 V_i \pi^4) S^2$$

where L is the length of the magnetotail, R_T is the radius of the magnetotail and V_i is the speed of the solar wind.

Figure 2 shows one of the smallest auroral ovals observed by the DMSP satellite. It was taken during a prolonged period of a large positive B_z value. Figure 3 shows an intense auroral substorm along an expanded medium size oval; this event occurred when the B_z component was negative. There is a considerable difference in the size of the oval and auroral activity, as expected from the above equation.

S.-I. AKASOFU

Geophysical Institute,
University of Alaska,
Fairbanks, Alaska 99701

1 Arnoldy, R., *J. geophys. Res.*, 76, 5189 (1971).
2 McPherron, R. L., Russell, C. T., and Aubry, M. P., *J. geophys. Res.*, 78, 3131 (1973).
3 Akasofu, S.-I., *Planet. space Sci.*, 22, 885 (1974).
4 Akasofu, S.-I., Perreault, P. D., Yasuhara, F., and Meng, C.-I., *J. geophys. Res.*, 78, 7490 (1973).
5 Lui, A. T. Y., Anger, C., and Akasofu, S.-I., *J. geophys. Res.* (in the press).
6 Sonnerup, B. U. O., *J. geophys. Res.*, 79, 1546 (1974).
7 Gonzaler, W. D., and Mozer, F. S., *J. geophys. Res.*, 79, 4186 (1974).
8 Burch, J. L., *Revs Geophys. space Phys.*, 12, 363 (1974).
9 Meng, C.-I., and Anderson, K. A., *J. geophys. Res.*, 79, 5143 (1974).

Natural halocarbons in the air and in the sea

ORGANOCHLORINE compounds are usually taken to be man made; yet methyl chloride has been demonstrated as a product of microbial fermentation[1] and of the smouldering combustion of vegetation (A. E. O'Keeffe, personal communication) and Zafiriou[2] has predicted its ubiquitous presence in the environment. Here I report measurements of methyl chloride and other halomethanes in the air and coastal waters of southern England between December 1974 and April 1975. These measurements confirm those of R. A. Rasmussen (personal communication) who found methyl chloride in rural air over the West Coast of the USA; they reveal methyl chloride as the dominant halocarbon of the atmosphere. The possible bearing of this discovery on the chlorine-catalysed destruction of stratospheric ozone is discussed.

Analysis was by gas chromatography using an electron capture detector which was operated at a temperature of 270 °C. This high temperature encouraged the otherwise sluggish reaction of methyl chloride with free electrons[3]. 5-ml air samples were analysed directly and water samples were analysed by taking 5-ml samples of nitrogen previously equilibrated with the water. The limit of detection for methyl chloride was 8.5×10^{-11} by volume.

Figure 1 compares the ambient concentration of methyl chloride and CCl_3F at Bowerchalke, southern England from
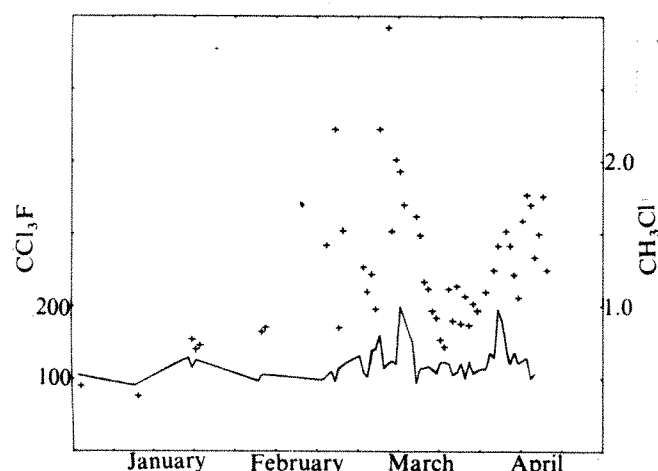
Fig. 1 Aerial concentration of CH_3Cl (+) and of CCl_3F (solid line) in parts per 10^9 and 10^{12} by volume respectively.

December 1974 until April 1975. The concentration of both gases varied with wind heading but methyl chloride was most abundant with air from the Atlantic whereas CCl_3F was most abundant with air from continental Europe. The mean concentration of methyl chloride over this period was 1.14×10^{-9} by volume. Table 1 lists the concentrations of halocarbons in seawater collected on the Kimmeridge ledges 30 miles south of the air sampling site. These inshore waters, rich in kelp, can be seen to carry large but variable amounts of halocarbons. Table 2 lists observations of methyl iodide in seawater during the voyages of the RV Shackelton in 1971 and 1972 and the RV Meteor in 1973. The first voyage covered the Northern and Southern Atlantic and Antarctic Oceans, the second the North Atlantic and the Caribbean Oceans. The table also includes some measurements of seawater concentrations of methyl iodide over kelp beds in

South-west Ireland. These latter measurements were during the summers of 1973 and 1974.

Methyl iodide has been found in all ocean waters so far examined and may be a common product of marine algae. The large kelp, particularly *Laminaria*, seem to specialise in the production of methyl iodide and water in the vicinity of the *Laminaria digitata* beds of South-west Ireland was found to contain one thousand times as much as is found in the open oceans. Other inshore algal beds, for example those of *Fucus*, showed no such increase. If methyl chloride is the product of the reaction between methyl iodide and the chloride ion of seawater[2], then the distribution of methyl chloride and perhaps also methyl bromide might be expected to follow a similar pattern to that of methyl iodide. So far only inshore waters have been examined for these compounds, where the concentration is so much greater than that which would be in equilibrium with the air that the sea is undoubtedly a strong source. If the halomethanes are the halogen gases discovered in the pioneering measurements of Duce and his colleagues[4,5] then the methyl halides are indeed ubiquitous.

The rate of reaction of methyl chloride with OH in the troposphere is such[6] that if this were the sole sink the residence time would be 0.37 yr. Applying this value to the observed mean concentration of 1.14×10^{-9} suggests a source strength of 28 Mt yr^{-1}. The industrial production of methyl chloride in 1973 was 0.35 Mt; this was used almost entirely as a chemical intermediate in the production of other compounds and did not reach the atmosphere as such. The atmospheric CH_3Cl is therefore almost certainly not a direct industrial emission. From the model introduced by Liss and Slater[7] some estimates of the seawater concentration neded to sustain a flux of 28 Mt yr^{-1} to the atmosphere can be calculated. This would suggest that with an aerial concentration of 1.14×10^{-9} the mean seawater concentration would need to be 1.9×10^{-8} ml of gas per ml of seawater. This requirement was reached on one occasion for the waters examined but an extended survey will be needed to determine the proportion of methyl chloride of marine origin. Approximately 1% of the chlorine content of vegetable matter is converted to methyl chloride during smouldering combustion. Grass and forest fires, stubble burning, and slash and burn agriculture are therefore potential sources also.

What is the significance of the high concentrations of methyl chloride for the present concern over the chlorine-catalysed destruction of stratospheric ozone? Unlike the freons, methyl chloride is comparatively unstable chemi-

Table 1 Halomethanes* in water from the seashore at Kimmeridge, Dorset, England

Date	Water temperature (°C)	CH_3Cl	CH_3Br‡	CH_3I
12.1.75	45	7.2 (2.7)†	2.0 (0.7)	1.3 (0.3)
8.3.75	42	5.9 (0.8)	1.5 (0.30)	1.2 (0.3)
9.4.75	42	21 (12)	3.9 (2.0)	2.8 (0.7)

*Concentrations are in ml of gas per ml of water $\times 10^{-9}$.
†Figures in brackets are standard deviations.
‡CH_3 Br confirmed by retention time only.

Table 2 Methyl iodide in surface seawater

Date	Site	Concentration*
1971–72	Open ocean Atlantic and Antarctic	135 (248)
1973	Open ocean Atlantic and Caribbean	138 (47)
1973	SW Ireland	$3.4 (1.8) \times 10^3$
1973	Kelp beds SW Ireland	$1.2 (0.9) \times 10^5$

*Concentrations as ml of vapour per ml of water $\times 10^{-12}$. Figures in brackets are standard deviations.

cally and the greater part of it would be destroyed in the troposphere. But methyl chloride has a source which is globally distributed and a residence time long enough to permit mixing in the troposphere. In these circumstances the observed ground level concentrations are likely to be representative and extend to the tropical tropopause. The proportion of chlorine conveyed to the stratosphere as methyl chloride to that conveyed as freon molecules will therefore be as their concentrations at the tropical tropopause. If other chlorine compounds are included, the present estimates suggest that less than 20% of the chlorine entering the stratosphere is in the form of freon[6]. Newell[8] calculates that the residence time for a molecule in the stratosphere between the 10 and 100 mbar levels is 2 yr. During this time nearly all of the methyl chloride entering the stratosphere will have been destroyed, releasing its chlorine. Because of their much greater stability the most likely fate of freon molecules entering the stratosphere is to return back to the troposphere. When this additional factor is taken into account the present production of chlorine in the stratosphere is mostly from naturally occurring methyl chloride. If the freons are allowed to accumulate in the atmosphere without hindrance eventually their concentration may reach levels which are hazardous. But at present they seem to constitute only a minor stratospheric chlorine source.

It is reasonable to assume that any man-made perturbation of a major compartment of the atmosphere such as the stratosphere is at the very least undesirable. In this debate, however, it is always assumed that ozone depletion alone is the serious problem and potential threat; the possible dangers of ozone accretion are in this context considered as irrelevant. An alternative view of the significance of odd nitrogen and odd chlorine in the atmosphere comes from considering the possibility that the cycling of gases by the biosphere is not merely passive but represents an active process concerned with homeostasis[9]. In the context of this hypothesis the large scale biosynthesis of methyl chloride and nitrous oxide, and the lesser production of the more potent methyl bromide, suggests a natural process for ozone depletion. Could it be that the biosynthesis of these compounds responds to some function of stratospheric ozone density and acts as a regulator?

I thank Drs S. A. Penkett and D. Johnson for the identification of methyl chloride, and Shell Research Ltd for support.

J. E. LOVELOCK

Bowerchalke,
Salisbury, Wiltshire, UK

[1] Cowan, M. I., *et al., Trans. Br. mycol. Soc.*, **60**, 347 (1973).
[2] Zafiriou, O. C., *J. mar Res.*, **33**, 73 (1975).
[3] Wentworth, W. A., Becker, R. S., and Tung, R., *J. phys. Chem.*, **71**, 1652 (1967).
[4] Duce, R. A., and Van Nahl, T. W., *J. geophys. Res.*, **70**, 1775 (1965).
[5] Moyers, J. L., Zoller, W. H., and Duce, R. A., *J. atmos. Sci.*, **28**, 95 (1971).
[6] Cox, R. A., Derwent, R. F., Eggleton, A. E. J., and Lovelock, J. E., *Atmos. Environ.* (in the press).
[7] Liss, P. S., and Slater, P. G., *Nature*, **247**, 181 (1974).
[8] Newell, R. E., Boer, G. J., and Kidson, J. W., *Tellus*, **26**, 103 (1974).
[9] Lovelock, J. E., and Margulis, L., *Tellus*, **26**, 2 (1974).

Terrain units in eastern Antarctica

APPROXIMATELY 12,000,000 km[2] of bedrock in Antarctica is inaccessible, except to remote sensing, beneath an ice sheet up to 4.5 km thick. The topographical and geological pattern of the subglacial continent has, consequently, been little investigated and no more than a few geophysical measurements have been obtained. The availability of continuous records of bedrock topography from airborne radio echo sounding, however, now

facilitates the calculation of statistics of terrain roughness, which can be used quantitatively to differentiate subglacial regions, to cluster similar units, and to indicate associations or characteristics related to geological factors[1].

A joint programme between the Scott Polar Research Institute (SPRI) and the US National Science Foundation (NSF) has obtained over 300,000 km of profiles in Antarctica since 1967 using pulse-modulated radar operating in the frequency range 35–60 MHz (refs 2–6). Some of these results have been used to define and interpret terrain units in eastern Antarctica.

Information on terrain roughness is available at two scales. A qualitative examination of detailed bedrock relief maps (contour interval 250 m) compiled following radio echo exploration (SPRI Antarctica Radio Echo Sounding Map Series A, sheet 3, 1974; scale 1 : 5,000,000) allows the delimitation of major physiographic provinces[7] (Fig. 1). Quantitative measures of the fine structure of surface roughness can be obtained from statistical analysis of the variation of relief within macroscale regions.

Vertical and horizontal variations in bedrock elevations have been calculated by the use of two simple statistics—the dispersion of elevations about the mean normalised to zero (D, standard deviation) and the autocorrelation distance (R_{xd}) calculated from the normalised autocorrelation function (with zero mean):

$$R_x = (n/n-k) \left[\left(\sum_{x=1}^{n-k} V_x \times V_{x+k} \right) \bigg/ \sum_{x=1}^{n} V_x^2 \right]$$

where V_x = discrete bedrock elevation; n = total number of elevations; k = lag of unit 1.5 km (the sample interval).

R_{xd} is defined as the lag to the jth % confidence level, usually taken as 95%. It is a distance over which roughness shows little or no statistical variation within given limits.

D and R_{xd} were computed for radio echo profile elevation data within macroscale regions of eastern Antarctica (Fig. 2). Two topographic groups can be identified, one comprising lowland areas with D values < 180 m (the Wilkes Basin and the basin in central eastern Antarctica) and a second, less homogeneous group of highland regions with values of $D \geqslant 200$ m (the Gamburtsev Mountains, subglacial portions of the Transantarctic Mountains and the massif in central eastern Antarctica).

The Gamburtsev Mountains fall into two distinct parts: one

Fig. 1 Major large-scale terrain regions of eastern Antarctica. T, Transantarctic Mountains; G and G*, Gamburtsev Mountains; H, highland massifs within central eastern Antarctica; W, Wilkes Basin; C and C*, central basin of eastern Antarctica; Mc, McMurdo Sound; *a*, terrain over 250 m; *b*, terrain below 500 m. (Both relate to present sea level.)
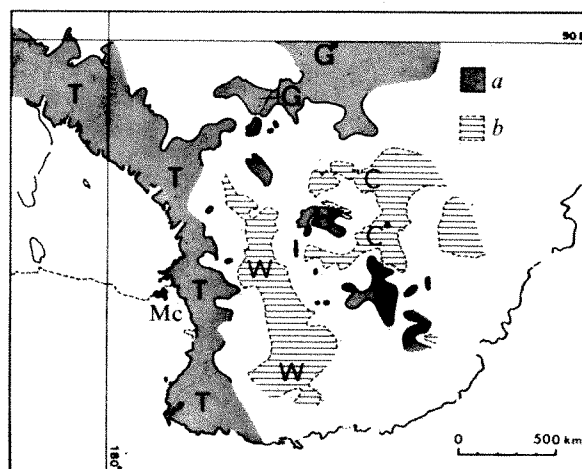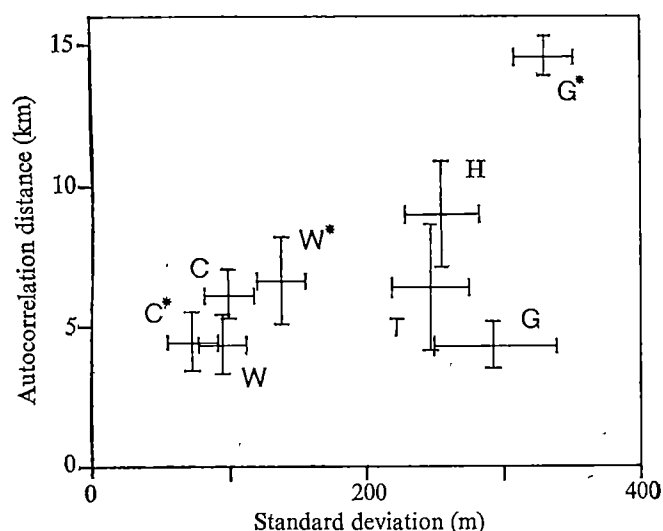
Fig. 2   Average terrain roughness characteristics of topographic units of eastern Antarctica. Bars indicate root mean squares of measurements. G and G*, Gamburtsev Mountains; H, central massif in eastern Antarctica; T, Transantarctic Mountains; W, central portion of Wilkes Basin; W*, marginal zones of Wilkes Basin; C*, northerly portion of central eastern Antarctic Basin; C, southerly portion of central eastern Antarctic Basin.

has a very high R_{xd} value indicating a smoother, more regular topographic surface, but is dissected by deep valleys to yield a commensurately high D value; the other, with a low autocorrelation distance, is typical of very rugged, rough mountainous terrain: values for D and R_{xd} are comparable with similar statistics obtained from other high latitude 'alpine' areas (for example, Alaskan ranges)[1].

Such contrasts within contiguous mountain zones probably originate from differences in the geological pattern. Unfortunately, no supporting geophysical information is available for this region. Nevertheless, it has been suggested that the Gamburtsev Mountains may have formed part of a large geosynclinal series deformed during a late Proterozoic–early Phanerozoic orogeny; an early Proterozoic deformational event has also been proposed[1]. The presence, however, of at least two distinct terrain provinces suggests a more complicated history of block formation and uplift. It may be that periods of epeirogenic reactivation in the Gamburtsev Mountains did not have uniform effects, either in time or place, leading to later diversification of terrain. The same result could also be the product of the differential stripping of an epiplatform cover developed during the Phanerozoic.

The Transantarctic Mountains and massifs within central eastern Antarctica show statistical similarities, but both are quite different from the Gamburtsev Mountains. The structural control of relief in both the exposed and the subglacial parts of the Transantarctic Mountains is partially attributable to the presence of the sub-horizontal sediments of the Beacon Supergroup and associated intrusive bodies[1,9,10]. Values of terrain roughness indicate that the central massif also falls within the same province of Phanerozoic sedimentation. Smaller R_{xd} values for the subglacial Transantarctic Mountains may be attributable to the more active erosional development of the mountains in the mid-Cainozoic, during the growth phase of the eastern Antarctic ice sheet[11], and to the later fluctuations of its margin in that zone[12].

The two major basinal areas of eastern Antarctica show strong similarities in terrain roughness, yet although they possess low D values they cannot be termed plain-like since they exhibit equally low R_{xd} characteristics.

The Wilkes Basin *sensu stricto* and its marginal areas were analysed separately. Results suggest that this depression (below present sea level), with only small scale surface irregularities along its axial core, is rimmed by a much rougher transition zone. Such

differentiation implies that the basin may contain a smoothing cover of Mesozoic and Cainozoic deposits which is absent from its flanks because of erosion. Calculations from isolated magnetic measurements over part of the Wilkes Basin provide limited evidence for the presence of a discontinuous, sedimentary sequence up to 6 km thick in places[13].

Similar, but slightly less marked, differences exist between two portions of the central eastern Antarctic basin. Since the margins of this basin show no distinctive roughness characteristics compared with the Wilkes Basin, any differences may result from a slightly greater surface dissection of a sedimentary mantle in the northern sector of the basin though it cannot be positively stated that sediments line the depression because of the absence of geophysical observations. In a small isolated basin beneath the Soviet Vostok base, 300 km to the south, however, seismic investigations of the upper crust indicate a thin layer of sediments[14].

These analyses may be important aids to the geological interpretation of inland areas of Antarctica and could be used as a primary reconnaissance tool for the location of critical areas worthy of detailed study using other geophysical techniques.

DAVID J. DREWRY

Scott Polar Research Institute,
Cambridge CB2 1ER, UK

1 Drewry, D J., thesis, Univ. Cambridge (1973).
2 Anonymous, *Polar Rec.*, 14, 211–13 (1968).
3 Evans, S , and Smith, B. M E , *Polar Rec.*, 15, 336–38 (1970).
4 Evans, S., Drewry, D. J., and Robin, G. de Q , *Polar Rec.*, 16, 207–12 (1972)
5 Evans, S , and Smith, B. M. E , *J. scient Instrum.*, Ser. 2, 2, 131–6 (1969)
6 Robin, G. de Q., Evans, S., and Bailey, J. T , *Phil. Trans. R. Soc.*, A265, 437–505 (1969).
7 Drewry, D. J , *Polar Rec.* 7, 359–374 (1975).
8 Grikurov, G. E., Ravich, M. G , and Soloviev, D. S , in *Antarctic Geology and Geophysics* (edit. by Adie, R. J.), 457–68 (Universitetsforlaget, Oslo, 1971)
9 Drewry, D. J , *ibid* , 693–704 (1971), in *Polar Geomorphology* (edit by Price, R. B.) and Sugden, D. M), 43–58 (Inst. Brit. Geogr. Special Publication No. 4, 1972).
10 McGregor, V. R., *J. Glaciol* , 4, 494–5 (1963).
11 Drewry, D. J., *J. geol Soc. Lond.* 131, 255–273 (1975).
12 Mercer, J H , *Bull geol Soc. Am.*, 79, 471–86 (1968).
13 Robinson, E. S , thesis, Univ. Wisconsin (1964).
14 Kapitsa, A P., *Inf. Byull. sov antarkt. Eksped.*, 19, 10–14 (1960).

Mechanochemical availability

THE thermodynamic functions 'availability' and 'maximum work', as they are usually defined[1-3], are generally inapplicable to systems that interest the biologist and electrochemist for the reasons shown below. Here I propose the redefinition and generalisation of both functions, and explicitly define a thermodynamic property, the 'mechanochemical availability', that provides a measure of the maximum usable mechanochemical work that can be released in the interaction of a contractile system with a source or sink of chemical potential of constant intensive state.

The 'maximum work' function

$$\Delta W \equiv \Delta U - T_r \Delta S \qquad (1)$$

is the work input along the singular process path that takes a system adiabatically and reversibly (isentropically) from its initial temperature, T_{system}, to the temperature of an interacting isothermal reservoir, T_r, (process 1–c) and that has all
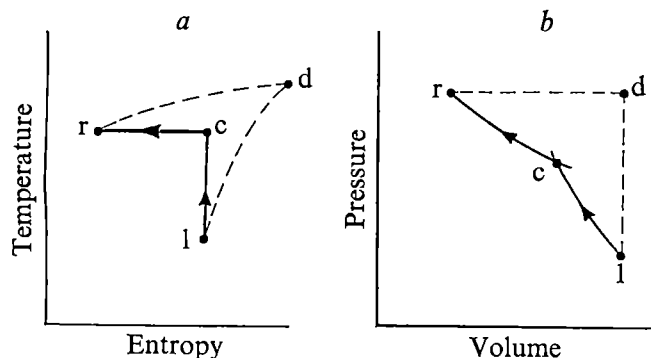
Fig. 1 T_1 may be greater than or less than T_r, but in this figure $T_1 < T_r$.

heat exchanges occurring subsequently and reversibly at T_r (process c–r) (Fig. 1a and b).

Thus

$$W_{1cr} = \Delta U_{1r} - Q_{1cr} = \Delta U - T_r \Delta S \equiv \Delta W \qquad (2)$$

The 'availability' is defined as

$$\Delta B \equiv \Delta W + P_r \Delta V \equiv \Delta U + P_r \Delta V - T_r \Delta S . \qquad (3)$$

When a system is capable of exchanging several work modes with its environment, equations (1) and (3) are of limited use or interest. To illustrate this deficiency, consider that an open mechanochemical system can engage in the following reversible work modes

$$W = \sum_i W_i = -\int_1^r P dV + \int_1^r f dL + \int_1^r \sum_i \mu_i dn_i \qquad (4)$$

which in turn equals

$$\Delta U - T_r \Delta S \equiv \Delta W \qquad (5)$$

when the system is constrained so as to transfer heat reversibly at T_r.

If the system is further constrained to operate at constant P_r, then

$$\Delta W = -P_r \Delta V + \int_1^r f dL + \int_1^r \sum_i \mu_i dn_i \qquad (6)$$

and

$$\Delta B = \int_1^r f dL + \int_1^r \sum_i \mu_i dn_i \qquad (7)$$

Equations (6) and (7) are deficient because they fail to separate the contraction work, $\int f dL$, from the chemical potential interchange, $\int \Sigma \mu dn$. Only the former term measures the useful mechanical work exchanged with the environment, yet the magnitude of this term is completely obscured in equations (6) and (7). In a mechanochemical system the 'availability of contraction work' is the significant or relevant quantity.

This deficiency may be removed by defining ΔW more generally:

"ΔW is the work input along that combination of reversible process paths which brings a system from its initial equilibrium state to a final state in equilibrium with an interacting reservoir of fixed state, said paths being such that all heat transfer occurs

at T_r and all matter transfer occurs at the potential of the reservoir (μ_{ri} or V_r)."

It now follows that the work input to a system so constrained is

$$W = \Delta U - Q = \Delta U - T_r \Delta S$$
$$= -\int_1^r P dV + \int_1^r f dL + \sum_i \mu_{ri} \Delta n_i \qquad (8)$$

As a further consequence, the 'maximum mechanochemical work' can now be defined as

$$\int_1^r f dL \equiv \Delta W_{mc} = \Delta U - T_r \Delta S + P_r \Delta V - \sum_i \mu_{ri} \Delta n_i \qquad (9)$$

if the system operates at P_r, and the 'mechanochemical availability' may now be defined as

$$\Delta B_{mc} \equiv \Delta W_{mc} - f_r \Delta L = \int_1^r f dL - f_r \Delta L \qquad (10)$$

or

$$\Delta B_{mc} \equiv \Delta U - T_r \Delta S + P_r \Delta V - \sum_i \mu_{ri} \Delta n_i - f_r \Delta L \qquad (11)$$

The significance of equations (9)–(11) is illustrated by Figs 2 and 3 which show the computation paths for ΔW_{mc} and
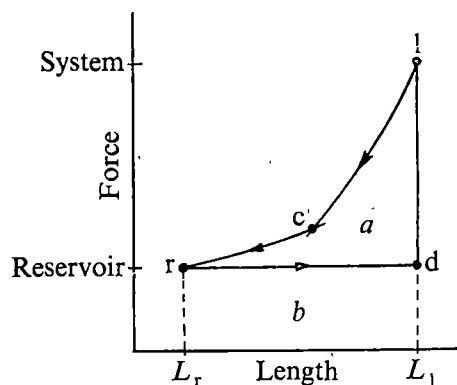
Fig. 2 Area $a = \Delta B_{mc}$; area $b = -f_r \Delta L$.

ΔB_{mc}, where 1 is any initial state of the system and r is the state of the system when in equilibrium with an interacting reservoir at T_r, P_r, μ_{ri} and f_r. Process 1–c is an isomolar reversible process which reduces the chemical potential of the system reversibly from μ_1 to μ_r. Process (c–r) then allows mass transfer at constant μ_r.

As can be seen from equations (10) or (11) the area (1–c–r–d–1) is equal to the mechanochemical availability.

At constant T_r and P_r, the cyclic integral of the Gibbs equation for a mechanochemical system:

$$\oint dU = T_r \oint dS - P_r \oint dV + \oint f dL + \oint \sum_i \mu_i dn_i \qquad (12)$$

reduces to

$$\oint f dL = -\oint \sum_i \mu_i dn_i \qquad (13)$$

from which it follows that area (1–c–r–d–1) in Fig. 2 equals

the negative of area (1–c–r–d–1) in Fig. 3, if only one component is exchanged with the reservoir, and

$$\sum_i [\text{areas } (1\text{--c--r--d--1})_i]$$

if i components are exchanged.

An analogous representation of the ΔB of equation (3), that is, the availability in a thermally activated expansion work system, is shown by the cycles (1–c–r–d–1) in Figs 1 and 2. The ΔB cycles always involve the properties of the work coupling and potential terms that appear in the Maxwell relations for the system. For a thermally activated expansion work system the relevant Maxwell relations in Jacobian form[4] are

$$[P, V] = [T, S] \tag{14}$$

whereas for a mechanochemical system at constant T and P they are

$$[f, L, T, P] = -[\mu_i, n_i, T, P] \tag{15}$$

The graphical representation of ΔB always consists of a cycle in which a constant n_i (or S) process is followed by a constant μ_i (or T) process, with the cycle completed by constant P and constant V processes or constant f and constant L processes. The area of the cycle is always less than or equal to zero.

By analogy the electrochemical availability may be defined as

$$\Delta B_{ec} \equiv \Delta U - T_r \Delta S + P_r \Delta V - \sum_i \mu_{ri} \Delta n_i - V_r \Delta_q \tag{16}$$

where V and q are voltage and charge, and a 'general availability function' may be defined as

$$\Delta B_g \equiv \Delta U - T_r \Delta S + P_r \Delta V - \sum_i \mu_{ri} \Delta n_i - f_r \Delta L + \sum_i F_{ri} \Delta X_i \tag{17}$$

where $\Sigma_i F_{ri} \Delta X_i$ represents the sum of all other reversible work forms expended against the interacting reservoir.

Is path (1–c–r) the maximum work path?

Curve (1–k–r) in Fig. 4 represents an arbitrary reversible mechanochemical process connecting the initial state (1) to the reservoir state (r). The area under (1–k–r) is clearly greater than area under the ΔW path (1–c–r).

Path (1–a–b–r), consisting of isomolar processes (1–a) and (b–r) and isopotential process (a–b), is so drawn that the area under (1–a–b–r) is equal to that under (1–k–r) (Clausius' theorem[5]).

For a constant temperature–constant pressure reversible
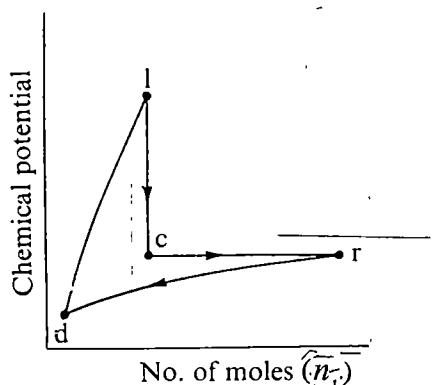
Fig. 4

Fig. 3
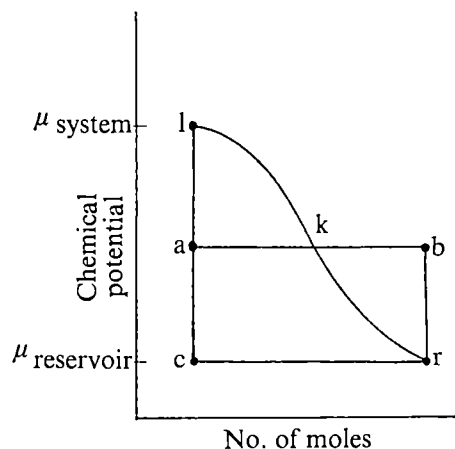
mechanochemical process whereby a system at 1 interacts reversibly with a reservoir at r

$$\int_1^r dU = T_r \int_1^r dS - P_r \int_1^r dV + \int_1^r f dL + \int_1^r \sum_i \mu_i dn_i \tag{18}$$

or

$$\int_1^r f dL = \Delta U_{1r} - T_r \Delta S_{1r} + P_r \Delta V_{1r} - \int_1^r \sum_i \mu_i dn_i \tag{19}$$

It follows from equation (19) that if the $\int \mu_i dn_i$ along path (1–k–r) is equal to the integral along (1–a–b–r), then the contraction works

$$\int_1^r f dL$$

for both paths are also equal. Now the work of process (1–a–b–r) exceeds that of process (1–c–r) by

$$\sum_i \left[\text{area } (a\text{--b--r--c--a})_i \right]$$

For process (1–a–b–r) to occur reversibly, however, it must absorb chemical work ($\mu_i dn_i$) at μ_b which is greater than the μ_r of the environment. A Carnot chemical pump[6] (the chemical work analogue of a heat pump) will therefore be needed to transfer this chemical work from the μ_r to the μ_b reversibly. The work input to this pump(s) is exactly equal to

$$\sum_i \left[\text{area } (a\text{--b--r--c--a})_i \right]$$

so that the net reversible work obtainable from the system (1) interacting with reservoir (r) is (−) the area under (1–c–r) plus ($\Delta U - T_r \Delta S + P_r \Delta V$), or $\int f dL$ along path (1–c–r).

Note added in proof: I thank Professor J. Keenan for pointing out that equation (9) is analogous to equation (55) of ref. 7.

M. V. SUSSMAN

Department of Chemical Engineering,
Tufts University,
Medford, Massachusetts 02155

1 Hatsopoulos, G. N., and Keenan, J. H., *Principles of General Thermodynamics,* 163 (Wiley, New York, 1965).

2 Wilkie, D. R., *Nature*, 245, 457–458 (1973).
3 Keenan, J., *Thermodynamics* (Wiley, New York, 1941).
4 Sussman, M., *Elementary General Thermodynamics*, 244–245 (Addison-Wesley, Reading, Massachusetts, 1972).
5 Zemansky, M., *Heat and Thermodynamics*, fourth ed., 168 (McGraw-Hill, New York, 1957).
6 Sussman, M., and Katchalsky, A., *Science*, 167, 47 (1970).
7 *The collected works of J. Willard Gibbs*, 1, 77 (Yale University Press, New Haven, 1948).

Table 1 Chemical analysis of the Egyptian rice hull ash	
Constituent	Weight (%)
SiO_2	91 28
TiO_2	0 12
Al_2O_3	2.82
Fe_2O_3	0.48
CaO	1.11
MgO	0.85
Na_2O	0.47
K_2O	1.79
Loss on ignition	0.23
Total	99.33

Wet preparation of 'water glass' from the hull ash of Egyptian rice

THE FAO and other organisations have suggested useful applications for the rice hull in agriculture[1]. But promotion of the use of the material for industrial applications was ignored. The use of the hull and its ash in the preparation of furfural and ceramic products has now been shown to be possible[1], but only the preparation of furfural from the hull has been established (in the USA and Italy) as a sound industrial process. Nevertheless, the furfural industry consumes only a small fraction of the material available, and research into other potential applications is obviously desirable. The work described here is an attempt at the wet preparation of water glass by decomposing the hull ash with sodium hydroxide solution at a moderately high temperature.

Water glass is normally prepared by fusing the appropriate quantities of sodium carbonate and sand at a temperature around 1,400 °C to produce a soda–silica glass This is converted into a viscous liquid through the action of water vapour under pressure[2]. The material has many applications in detergents, adhesives, ceramics, paper, textiles and other industries.

The annual world production of rice is $\sim 250 \times 10^6$ t, of which about 10^6 t are produced in Egypt. The hull constitutes about 20% of the rice grains. The material has a very low bulk density (about 0.13 g cm^{-3}), and thus occupies

a very large space. It also has a high silica content which makes it of little value as food for animals. The hull is used as a fuel, especially in some rice mills. When well burnt it leaves a whitish fluffy voluminous ash which retains the original shape of the hull, and consists chiefly of silica in an amorphous form[1].

For the determination of the ash content of the hull and the thermal behaviour during ignition, thermogravimetric (TG), differential TG and differential thermal analysis (DTA) graphs were established on a Model OD-102 Hungarian-made Derivatograph.

Figure 1 illustrates TG, DTG and DTA curves for a 340-mg sample of the hull. The sample was heated from room temperature to 1,020 °C in an atmosphere of air at a heating rate of 10 °C min^{-1}, using alumina as the inert reference. The DTA curve shows an initial endothermic peak at about 130 °C corresponding to the loss of adsorbed water. This is accompanied by a similar endothermic DTG peak at a slightly lower temperature (115 °C), and a loss in weight (represented by the TG curve) of about 4.4% of the initial sample weight. The thermal decomposition of the hull is characterised by two exothermic DTA peaks at about 350–400 °C and a composite endothermic DTG peak with the principal apex at 330 °C. These peaks are probably characteristic of cellulose and hemicellulose which are the major constituents of the organic part of the hull. Corresponding to these reactions there was an abrupt loss in weight of about 36.6% of the initial sample weight. The decomposition of the remaining part of the hull was gradual and was completed at about 942 °C after which the weight of the residual ash became fixed at 60 mg (about 18% of the original sample weight).

Complete chemical analysis of the ash remaining after the complete burning of the rice hull was carried out by the method described by Riley[3]. Table 1 presents the percentages of its various constituents, the main one being silica (91.28%). On washing the ash with 0.5 N HCl overnight the silica content rose from 91.28% to about 95.5%. This is chiefly because of the preferential leaching of alkalis and other basic constituents in the form of chlorides.

The decomposition of the ash was carried out in a bomb of 250 ml capacity, made of stainless steel and placed in a thermostatically controlled kiln. In each experiment 5 ml of distilled water was mixed with 5.0 g of a mixture of ash and sodium hydroxide, which had a silica–soda ratio of 2 : 1. The bomb containing the mixture was then placed in the kiln, which was previously adjusted to the required temperature, for the required time. After the experiment was completed, the bomb was taken out of the kiln, cooled to room temperature and opened The contents were then discharged in a 250-ml beaker, using 30–40 ml water and quickly filtered through sintered glass For determining the degree of decomposition of the ash, the filtrate was analysed for its silica content. Figure 2 illustrates the time dependence of decomposition at 100, 125, 150, 175 and 225 °C. The fraction decomposed increases with time and temperature, the reaction being fast in its early stages and slower as complete decomposition

Fig. 1 Ash content of the hull of Egyptian rice.
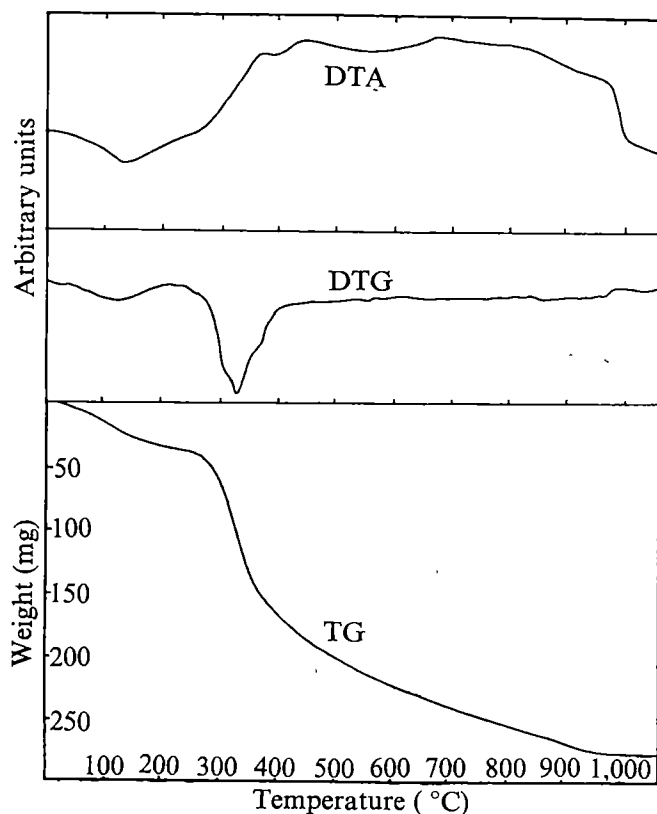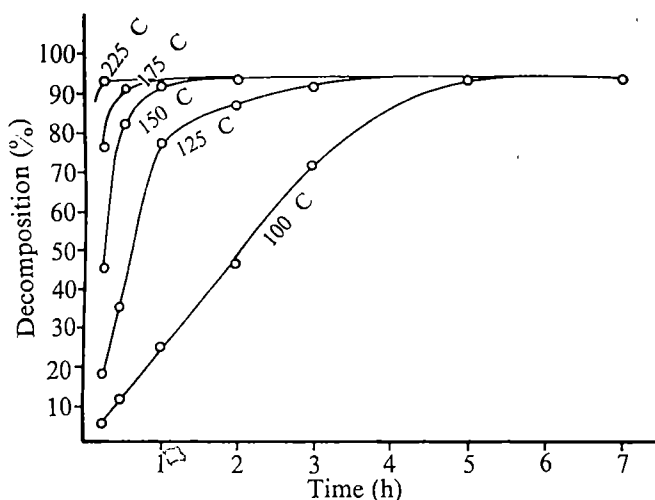
Fig. 2 Time dependence of decomposition of the raw ash at different temperatures.

is approached. Nearly complete decomposition is achieved after 5, 4, 2, 1 and less than 0.5 h at temperatures of 100, 125, 150, 175 and 225 °C, respectively.

For comparison raw ash, acid washed ash and finely powdered quartz and fused silica (−150 mesh) were subjected to a decomposition experiment at 150 °C for 1 h. The results are given in Table 2. The raw ash is much more easily decomposed than the other forms of silica. The acid treated ash is even more easily decomposed. This can be attributed to the partial leaching of cations such as Al^{3+}, Ca^{2+} and Mg^{2+}, which are known to suppress the dissolution of silica in alkali solutions by precipitating it as silicates[4].

The explanation of the decomposition of the ash by NaOH, can be simplified by taking the relatively simple structure of pure silica as a first approximate model for the more complicated structure of the ash. The effect of other cations can be treated separately.

In all forms of silica, silicon is known to be tetrahedrally coordinated to four oxygen atoms. Within the material, the coordination of oxygen and silicon of the tetrahedral units is satisfied. At the bounding surface, however, silica may expose incomplete tetrahedra and the surface ions will strive to complete their coordination. In contact with aqueous solutions the exposed ions will add hydrogen or hydroxyl ions so that the surface is ultimately covered with Si–OH groups. The hydrogen of the Si–OH group is ionisable and may, in favourable conditions, leave the silica surface in a negatively charged state. According to the details of the structure, there are four ways in which these Si–OH groups may be exposed at the surface[5,6] (Fig 3). These four types show enormous variations in resisting attack by aqueous solutions. Although type I is very unstable in the presence of water, type IV is completely unreactive to alkalis[5]. A demonstration of the importance of the surface structure is provided by low quartz. The basal plane shows a rate of attack by aqueous solu-

tions a hundredfold greater than the prism faces. The former is known to present type II exposure whereas the latter alternates between type II and type III, chiefly existing in the more resistant type III (refs 5 and 6). Noncrystalline silicates such as glasses and rice hull ash would be expected to present surfaces in which the four structural types exist.

In the rice hull, cellulose, hemicellulose and other carbonaceous material, which interlink the structural silicate units, are converted into CO_2 and H_2O by burning. As these materials constitute some 80% of the hull, the ash would be expected to be left in a state of polymerisation from which it can be more easily decomposed than pure forms of silica. This is, indeed, the case (Table 2).

In fact, simple shaking of ash with water at room temperature for a few minutes, brought about the maximum solubility of silica in water (about 0.01%). This supports the view that the structural units in the ash contain a high proportion of the easily soluble monomeric and dimeric units of silica.

The presence of polyvalent cations is known to suppress the dissolution of silica in alkaline solutions[4]. This is perhaps the reason why the maximum decomposition
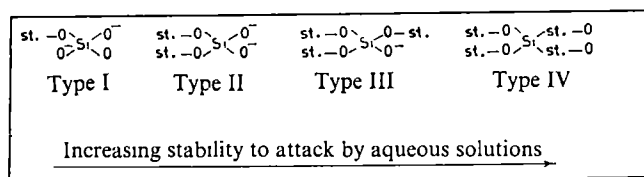
Fig. 3 The four possible exposures of the silica tetrahedra. st.=Structure.

achieved was about 94%. An experiment carried out on ash which has been previously washed with 0.5 N HCl, to remove some of the polyvalent cations, gave a maximum degree of decomposition of about 97% in the same conditions (see Table 2).

Thus, the structural state of the rice hull ash makes it superior to other forms of silica in the wet preparation of water glass. The economy of the process lies mainly in the relatively lower temperature at which reaction proceeds. Moreover, the hull—an inconvenient byproduct—can find a useful industrial application.

AHMED M. EL BOUSEILY

Geology Department,
Alexandria University,
Alexandria, Egypt

TAYSEIR M. EL SHAMY

Chemistry Department,
Tanta University,
Tanta, Egypt

1 Abu Shady, A. M., *et al*, *Proc. First Rice Conf.* (Egyptian General Establishment of Mills, Cairo, 1970).
2 Vail, J. G , *Soluble Silicates* (Reinhold, New York, 1952).
3 Riley, J. P , *Analyt. Chem* , 19, 413–428 (1958).
4 Iler, R. K , *The Colloidal Chemistry of Silica and Silicates* (Cornell University Press, Ithaca, 1955).
5 Ernsberger, F. M., *J. Phys. Chem. Solids.*, 13, 347–351 (1960).
6 Sosman, R. B., *The Properties of Silica* (The Chemical Catalog Company, 1927).

Table 2 Decomposition of raw ash, acid washed ash and finely powdered quartz and fused silica with NaOH solution under pressure

Type of silica	Ratio of SiO_2–Na_2O	Ratio of water–solids	Temperature (°C)	Time (h)	Decomposition (%)
Raw ash	2:1	1:1	150	1	92.7
Acid washed ash	2:1	1:1	150	1	96.8
Quartz	2:1	1:1	150	1	7.0
Fused silica	2:1	1:1	150	1	60.7

Is the vegetation of continental Antarctica predominantly aquatic ?

WE have suggested[1] that Antarctic lakes offer a more favourable physical environment to certain species of moss than the surrounding land. We now present evidence in

support of the wider thesis that in certain areas of Antarctica most of the plant biomass occurs in aquatic habitats. In the austral summer 1973–74 we found more mosses and algae growing aquatically than terrestrially in the Ablation Valley area of Alexander Island (Fig. 1). The area is relatively free from ice and has a climate similar to the inland ice-free areas of continental Antarctica[2], although conditions are not quite so arid because of frequent intrusions of oceanic weather systems from the Bellinghausen Sea. Extensive terrestrial plant cover was, however, restricted to seven discrete patches of moss, lichen and algae, totalling 2,300 m² in about 40 km² of ice-free ground searched on foot. The patches were on north-facing slopes where groundwater welled up continuously during the short summer. Widely scattered, very small moss cushions also occurred between and beneath stones in a few other damp and wet places. The availability of water and soil instability seemed to be the two most important factors restricting terrestrial plant distribution.

Many small pools, free from ice in summer, lay on ice-cored unstable moraines which bordered George VI Sound (Fig. 1). Although many pools may exist for only a few years, 95% supported a rich algal flora and associated microfauna including the calanoid copepod *Pseudoboeckella* sp.. A few pools also contained moss.

Ponds, 500–10,000 m² in area, lay between the scree-clad mountains and the moraines of ice shelf of the Sound. They became partly or completely free from ice for a short period in summer. The turbidity of the waters varied from less than 10 to more than 90% transmission per m as a result of the suspended glacial clay. In all ponds with a light transmission of more than 50% m⁻¹, luxuriant moss growth covered large areas from 0.5 to 9.0 m depth. Stems grew 30 cm in length. *Campylium polygamum* and *Dicranella* sp. were dominant. *Distichium capillaceum*, *Bryum algens* and another *Bryum* sp. were also found. Cover was estimated during SCUBA dives and varied from 40 to 80% in the 0.5 to 5.0 m depth zone and 20 to 50% overall. Total moss cover in these clearer ponds was 3,500 m² in an area of 10,000 m². Most of the remaining area was covered by rich

Fig. 1 Outline map of Alexander Island and its position in Antarctica. ———, Definite ice coastline; - - - -, approximate ice coastline. Ice shelf, stippled area. Front of ice shelf, dashed area.

algal felts growing up to 10 cm thick. Less luxuriant algal felts occurred in many of the more turbid ponds.

Three large (1.5–6 5 km²), deep (50 to more than 117 m), ice-dammed lakes lay in the main valleys. They were permanently frozen, ice cover ranging from 4.0 to 4.5 m thick in winter, 2.5 to 3 0 m thick in summer. No moss was seen in five dives covering about 10,000 m² of the bottom of the lake in Ablation Valley, and extending to 15 m depth. The only vegetation observed was a thin film of algae on the occasional rock jutting out of a silt floor. Silting alone is unlikely to have prevented moss growth because the water contained less suspended clay than some of the ponds in which mosses were abundant. The dives were carried out near the shore where the ice was more than 5 m thick and snow cover persisted for most of the summer. This may have reduced light levels below that required for moss growth. Further out in the lake where the ice was snow free and thinner, light levels may have been insufficient due to the deeper water. Mosses may have been present in other areas of this 6.5 km² lake, particularly near the north-west shore where the snow cover disappeared very early in summer. It should be noted that mosses have been found at 35 m depth in other permanently frozen lakes[3].

Contrary to our findings in the South Orkney Isands and South Georgia[1] mosses grew well in shallow water where ice scour could occur. Most of the moss must be frozen each winter. This must bring into doubt our earlier conclusion that mosses are unable to withstand ice scour. But the way ice cover melts on the Ablation ponds may differ so that ice scour is not a significant factor.

Further evidence for the predominance of aquatic vegetation in continental Antarctica is available. Rich benthic floras have been discovered in Lake Bonney[4], South Victoria Land (77°S, 161°E), lakes in the Bunger Hills[3] (66°S, 101°E) and in the Schirmachervatna[5] (71°S, 11°E). These are very arid areas free from ice where terrestrial vegetation is extremely limited.

We conclude that in such areas the vegetation may largely be confined to aquatic habitats. Consequently biologists should look more closely at aquatic habitats when carrying out surveys in continental Antarctica.

We thank Dr S. W. Greene and B. G. Bell (Institute of Terrestrial Ecology) for identifying the mosses.

J. J. LIGHT
R. B. HEYWOOD

British Antarctic Survey,
Monks Wood Experimental Station,
Abbots Ripton, Huntingdon PE17 2LS, Cambridgeshire

[1] Light, J J., and Heywood, R. B., *Nature*, 242, 535–36 (1973).
[2] Heywood, R B., *Bull. Br. Antarct Surv*, 29, 35–65 (1972)
[3] Korotkevich, V. S., *Inf. Byull. Sov. Antarkt. Eksped*, 3, 91–98 (1958)
[4] Taylor, G , *With Scott. The Silver Lining*, 136 (Smith Elder, London, 1916).
[5] Savich-Lyubitskaya, L. I , and Smirnova, Z. N., *Inf. Byull, Sov. Antarkt. Eksped*, 49, 33–39 (1964).
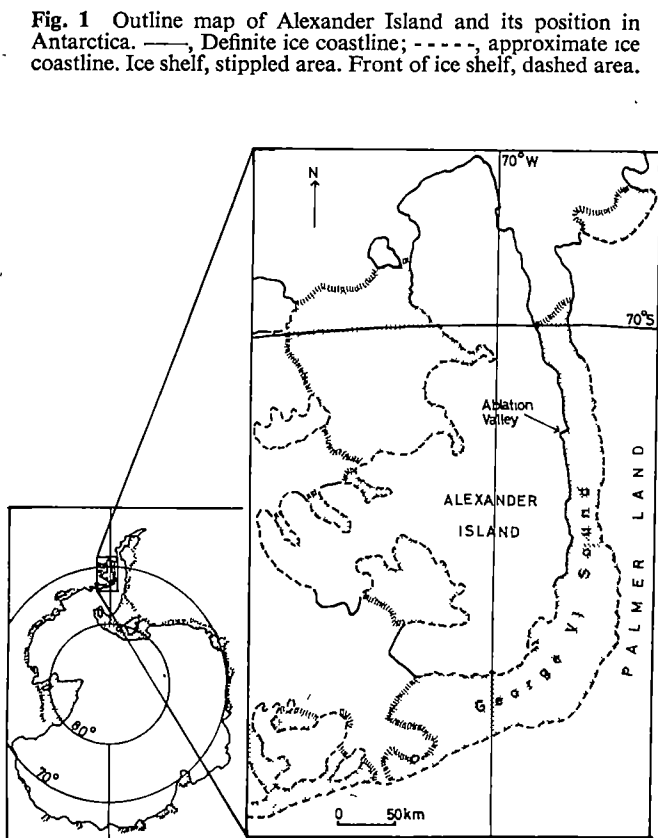
Form and function in corrugated insect wings

SELECTION is likely to have resulted in the evolution of insect wings which combine aerodynamic efficiency with a rotational moment of inertia about the wing base small enough to reduce as far as possible the energy expenditure involved in their repeated accelerations. Their construction has to leave them stiff enough to remain aerodynamically efficient when under inertial or aerodynamic load, and free from buckling, however light they become. Insect wings are very light structures—11 g m⁻² in the dragonfly *Aeschna cyanea*, 16.7 g m⁻² in *Locusta migratoria*[1] and 7.4 g m⁻² in *Tipula* sp.[2].

Hertel[1] has drawn attention to the corrugated chordwise profile of the anterior part of dragonfly wings, to which he attributed much of the wing stiffness, and commented on the
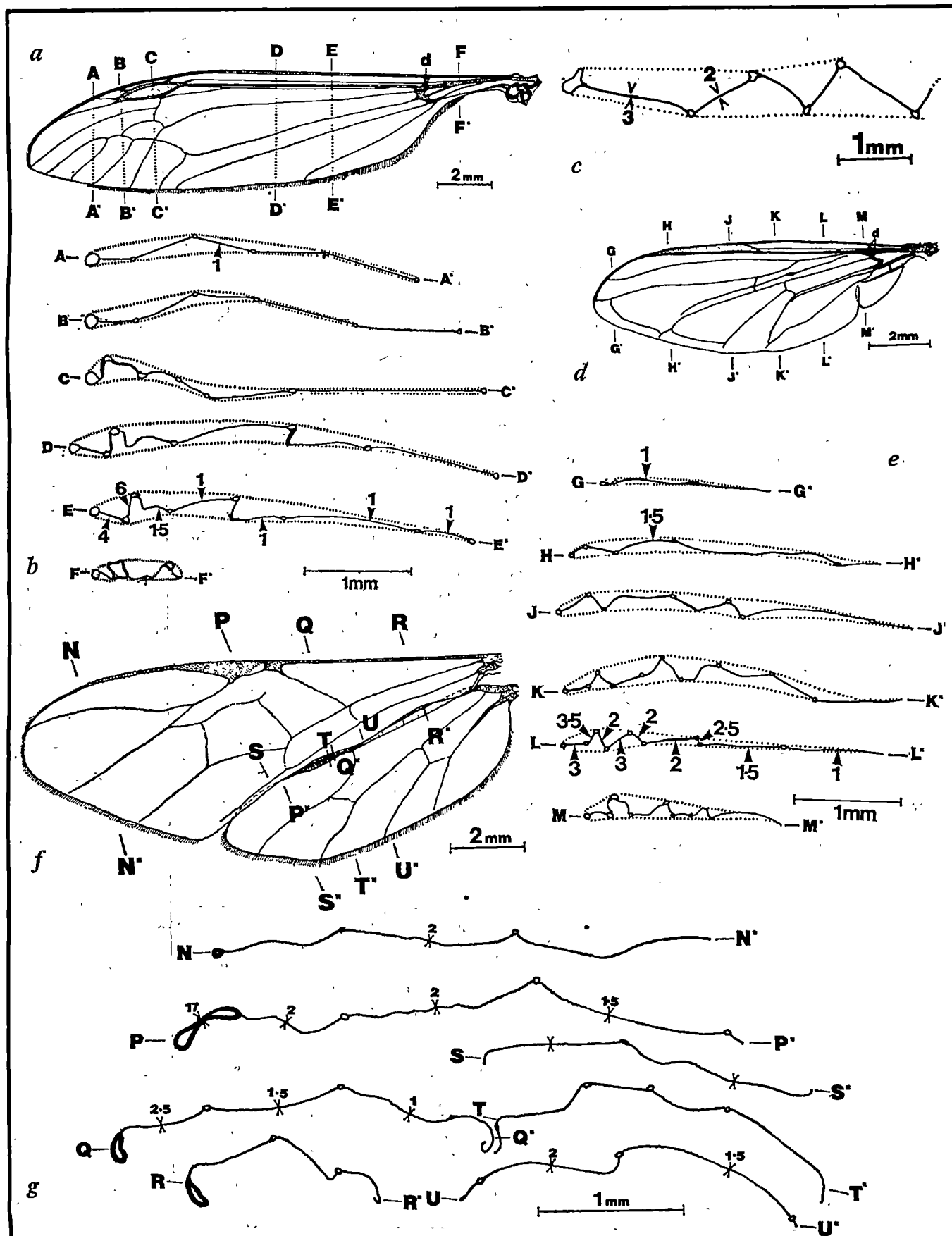
Fig 1 *a*, Left wing of *T. oleracea* (crane fly). d, Cuticular bar which reinforces the corrugation. Section letters apply to *b*. *b*, Chordwise sections of *T. oleracea* wing. Tubular veins to scale, membrane thickness shown diagrammatically. Some measured membrane thicknesses are shown (μm). Dotted lines, section envelopes. *c*, A section of the anterior portion of the chord of an *Aeschna cyanea* forewing (anisopteran dragonfly), redrawn from Hertel[1]. *d*, Left wing of *Syrphus balteatus* (hoverfly). d, Cuticular bar reinforcing corrugation. Section letters apply to *e*. *e*, Chordwise sections of the wing shown in *d*. *f*, Left wings of *Ophion luteus* (ichnuemon). *g*, Chordwise sections of the wings shown in *f*.

similarity between the disposition of the spars which form the web between upper and lower skins of an aeroplane wing, and the corrugated section of the *Aeschna* wing. In an aeroplane wing with a double skin separated by struts, however, it is the function of the struts to prevent the deformation of the smooth aerofoil profile presented by the skin, as is necessary at the higher Reynolds numbers appropriate to aircraft. In insect wings there is no enveloping 'skin' and any aerofoil properties must be sought in the corrugated wing sheet itself, operating at much lower Reynolds numbers at which the effect of corrugation on aerofoil lift and drag may not be so deleterious.

Figure 1 shows some series of chordwise sections of the wings of three insects: two dipterans (*T. oleracea* and *Syrphus balteatus*) and a hymenopteran (*Ophion luteus*). These were prepared by hand grinding thick slices cut from epoxy resin blocks, in which whole wings had been embedded, to approximately 10 μm thickness with wet carborundum paper. The embedding did not alter the corrugated form of the wings from their configuration in life; the corrugations were easily visible with either a stereomicroscope or a scanning electron microscope, where no embedding has been done. Corrugation is pronounced in both flies in most chordwise sections except those very near the wing tip. It tends to be deepest at about the one-third chord point from the leading edge, which has itself a notably rounded profile, especially in the *Ophion* wing. A somewhat similar architecture is seen in the wing of the beetle *Cicindela hybrida*[3]. Corrugation is less pronounced in *Ophion*, but the leading edge is well rounded, thickened and rigidly bent downwards.

Some comparisons of the mechanical properties of more regularly folded or tubularly reinforced beams may be relevant to a consideration of the load-bearing characteristics of insect wings. It is clear from Fig. 1 that insect wings are not beams of regular cross section, or of uniform width. They are corrugated beams of a sort, and I have therefore estimated some of the mechanical properties of a few beams of corrugated or tubulated cross section, the proportions of which are at least of the same order as those in some insect wings with respect to corrugation width, depth, membrane thickness and tube inner and outer radii. Experimental tests with metal scale models reveal that for small deflections corrugated beams which are loaded as cantilevers will behave as is predicted by simple beam-bending theory. Figure 2 summarises the dimensions of the beams which were compared, and Table 1 provides average

values of these dimensions for chordwise profiles of seven species of insects. It is hoped that these values will convey some impression of the relative cross sectional conformations of the wings of a diversity of insect species. Simple description of these profiles is otherwise difficult.
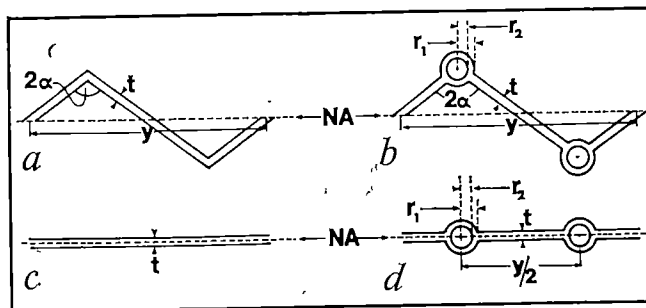
Fig. 2 *a–d*, Diagrams of parts of the cross sections of four beams the mechanical properties of which are compared in the text. *a* and *b* each show one corrugation repeat unit. NA, neutral bending axis. All other letters are those used in equations (1) to (4) in the text.

In making comparisons between regular beams, the following values were selected. The notation is as in Fig. 2, and the values apply to beams *a–d* in Fig. 2, as appropriate. Width of beams along neutral axis, $w = 1$ cm; length of beams $l = 1$ cm; $t = 1.2$ μm; $2\alpha = 112°$; $y = 0.05$ cm; $r_1 = 10$ μm; $r_2 = 4$ μm. The beams were all assumed to be made of a material similar to insect cuticle with a density of $\rho = 1.1$ g cm^{-3} and Young's modulus $E = 10^{10}$ N m^{-2} (ref. 4). Table 2 gives values of tip deflection D, maximum stress σ_M, second moment of cross sectional area I_x, mass m and rotational moment of inertia I_r about an axis passing through one end of the beam perpendicular to its length and parallel with the neutral axis and chord (Fig. 2).

The following relationships were used to compute the mechanical properties of the four beams *a–d* in Fig. 2. *F* is the force applied to the free end of the beams. $D = Fl^3/3EI_x$, $\sigma_M = Flz/I_x$, $m = \rho l A_x$ where z is the maximum distance of any part of the cross section from the neutral axis and A_x is the surface area of beam cross section. Second moments of cross

Table 1 Average values of beam dimensions for chordwise profiles in seven species of insect

Insect species	Average dimensions					Section position (mm)		
	y (cm)	t (μm)	2α (degrees)	r_1 (μm)	r_2 (μm)	A	B	
Aeschna cyanea (dragonfly) forewing	0.15	2.5	102	60	50	12.8	0.27	*
Chrysopa signata (lacewing) hindwing	0.07	1.3	160	15	12	9.2	0.67	
Panorpa communis (scorpion fly) forewing	0.03	1.0	150	20	16	10.8	0.77	†
Arctia caja (moth) forewing	0.40	5.0	146	70	60	25.0	0.86	
Ophion luteus (ichneumon) forewing	0.15	2.0	158	15	12	2.6	0.37	
Tipula oleracea (crane fly) forewing	0.10	1.5	128	20	17	15.7	0.81	
Syrphus balteatus (hoverfly) forewing	0.05	1.2	112	10	4	3.9	0.41	‡

*Averaged over the corrugations in the anterior third of the chord only.

†The tubular veins are here represented by veins of U-shaped section 40 μm deep and 32 μm wide

‡These values were used in the comparisons of various regular beams. Let straight line chord length $= C$ and number of veins in the section $= N$. Average y was estimated as $y = 2(C/N - 1)$, counting the trailing edge as a 'vein'.

Average t was that of the thickness of the inter-vein membranes, measured at the mid points (chordwise) of each.

A, Distance along span of chordwise section from wing root (mm); B, fraction of total wing length which A represents.

r_1 and r_2 were average outer and inner radii for all actual veins in the cross section. Where a vein is not circular in section, the average of its maximum and minimum radii was used.

2α represents the average angle included by all pairs of membranes where they merge with a vein.

Table 2 Parameters of regular beams

Beam section type	Deflection (D) (cm)†	Mass (m) (g)	Maximum stress (σ_M) (N m^{-2})	I_x (cm^4)	I_r (kg m^2)
a	0.97×10^{-3}	1.45×10^{-4}	2.48×10^5	3.43×10^{-9}	4.83×10^{-12}
b	0.32×10^{-3}	2.41×10^{-4}	8.64×10^4	1.03×10^{-8}	8.03×10^{-12}
	$\gg 0.11^*$	1.32×10^{-4}	8.33×10^7	1.44×10^{-13}	4.40×10^{-12}
c	0.11^*	2.37×10^{-4}	3.28×10^6	3.05×10^{-11}	7.90×10^{-12}

$$\text{where } I_r = \int_{r=0}^{r=l} \rho A_x r^2 dr = \frac{\rho A_x l^3}{3} = \frac{m l^2}{3} \qquad (5)$$

*Deflections here will be increased by deformation under the beams' own weights, which will also increase the stress σ_M.
†Stress and deflection are expressed per 10^{-5} N applied at the free beam tip. The other ends of the beams are all fixed rigidly.

sectional area, I_x were obtained from the expressions:
For beam a:

$$I_x = \cot^3\alpha/48 \, (y^3 t \sec\alpha + 4 y t^3 \sec^3\alpha) w/y \qquad (1)$$

For beam b:

$$I_x = \{(h^3/3b^3)(c^4 + 4b^3 + 6b^2 c^2 + 4b^3 c) + \pi[((r_1^4 - r_2^4)/2) + d^2(r_1^2 - r_2^2)]\} w/y \ldots \qquad (2)$$

where $b = [(y \csc\alpha/4) - r_1 - (t \tan\alpha/2)] \csc\alpha$; $h = b \sin\alpha \cos\alpha$; $c = t \sec\alpha$.
For beam c:

$$I_x = wt^3/12 \qquad (3)$$

from Roark[5], and for beam d:

$$I_x = \left[\frac{(y - 4r_1)t^3}{12} + \frac{\pi (r_1^4 - r_2^4)}{2} \right] \frac{w}{y} \qquad (4)$$

We thus see that the introduction of corrugation is associated with scarcely any weight penalty (comparing beams a and c) although there are immense reductions both in deflection and in the maximum stress experienced for a given loading. The tubes, when added to the beam of corrugated cross section, increase its mass by a factor of 1.66, but result in a decrease in maximum stress by a factor of 2.87. Inclusion of tubes in a plane section (beam d) results in almost as great a mass as in the corrugated, tubed section (beam b), but beam d still deflects 337 times as far as beam b for a given end load, is 38 times as highly maximally stressed and yet its rotational moment of inertia I_r is only 2% less than that of beam d. What then is the function of the tubes? In a real insect wing, these tubes (veins or nervures) often carry haemolymph or serve as conduits for nerves and tracheae. In a corrugated beam with a very thin web there is in bending a tendency to collapse suddenly and catastrophically by buckling at the folds. The inclusion of tube at these positions reduces the likelihood of this form of failure.

Insect wings show irregular depth of corrugation, both chordwise and spanwise. Corrugation tends to be deeper in the anterior half of the wing, and probably reflects the chordwise distribution of aerodynamic forces. In the use of corrugated structures, there is also the problem of preventing the folds from opening out when under load. In both fly wings in Fig.1a and d there are stout chordwise bars (d) at the thoracic end of the corrugations which rigidly connect them in much the same manner as the end diaphragms in a corrugated roof to a building[6]. At the outboard end, as is seen in sections A–A[1] or G–G[1], the corrugation is hardly detectable. As such a nearly flat profile can permit virtually no further extension in a chordwise direction,

any corrugation in the wing between this flat region and the more proximal diaphragm (d) is made unable to flatten out, and the wing should retain its stiffness.

Unlike the double-skin aircraft wing, supported internally by a corrugated strut system, however, plainly corrugated wings like those of insects will have very little resistance to torsional deformation. Norberg[7] has shown, however, in dragonflies that some torsional pitching of the wing during acceleration may allow increased flight speeds, under the influence of the pitching moment of the pterostigma, and that the natural axis about which the wing twists most easily is not far from the axis of fore-and-aft mass balance of the wing. Torsional deformation of inertial and aerodynamic origin may well be very small.

CHRISTOPHER J. C. REES

Department of Biology,
University of York,
Heslington, Yorkshire YO1 5DD, UK

1 Hertel, H., *Structure, Form, Movement,* ch. 3, 78-87 (Reinhold, New York, 1966).
2 Sotavalta, O., *Ann. Zool Soc. Fenn 'Vanamo',* 15, 1-67 (1952)
3 Kaufmann, T (1960), *Diss. Nat. Fak. Univ. München* (unpublished) cited by Nachtigall, W., in *Insects in Flight,* 121 (McGraw-Hill, New York, 1974).
4 Jensen, M., and Weis-Fogh, T., *Phil. Trans. R Soc.,* B245, 137-169 (1962).
5 Roark, R. J., *Formulas for Stress and Strain* (McGraw-Hill, New York, 1965).
6 Morgan, W., *Elements of Structure An introduction to Principles of Building and Structural Engineering* (Pitman, London, 1968)
7 Norberg, R. A , *J. comp. Physiol.,* 81, 9-22 (1972)

Improving one's touch

DRAWING the finger tips across a smooth surface usually induces sensations of pressure, warmth or cold, and roughness or smoothness. Any abrupt discontinuity is easily felt: it has been claimed that eminences no higher than 0.001 mm etched on to smooth glass can be detected, provided some movement of the finger tips is permitted[1]. We describe here an effect which does not seem to have been reported previously, even though we think that it must be familiar to certain craftsmen.

The effect is easily demonstrated. First, rub the fingers of one or both hands back and forth across a fairly smooth surface, such as polished wood. Note the accompanying tactile sensations. Next, place a piece of paper under the fingers and rub the surface as before, moving the paper with the fingers It will be found that any gradual undulations in the surface which were hitherto undetected are now perceptible. (With practice and concentration it becomes possible to detect similar undulations without using the paper, but this is seldom as quick and easy.)

We have examined the effect in experimental conditions. A series of steel blocks, each 7.6×7.6 cm, was used. The upper surface of each block was ground smooth and flat and contained a central, raised, rectangular strip 3 mm wide. The height of the central strip varied from block to block. The central strip on each block was converted to a

gradual undulation by covering the upper surface with smooth card (0.5 mm thick) secured firmly at the edges.

Eleven subjects attempted to detect the orientation of the covered central strips by rubbing the surfaces with the fingers of one hand. On half the trials fingers alone were used, on the remaining trials a piece of writing paper was held under the fingers. The use of fingers alone compared with paper was counter-balanced across trials, as was the initial orientation of the hidden strip. Rubbing movements were made towards and away from the body; the subject was allowed to rotate the block through 90° whenever he chose. The subjects could not see the surfaces they were feeling. Twelve trials were carried out for each condition.

Performance at this task was so good, particularly when the paper was used, that we shall describe only the results obtained using the most difficult block, in which the central strip was 0.0127 mm high. Without the masking paper the orientation of the central strip on this block was correctly detected on only 59.8% of trials overall. Using the masking paper 79.5% of trials were correct. The difference in performance in the two conditions is statistically significant ($F=8.2$, $P<0.05$).

It has been established[2] that the skin contains more than one type of receptor responsive to mechanical deformation. This finding suggests a possible explanation for the effect described. If rubbing an undulating surface commonly induces more than one kind of sensory input, then a highly sensitive system, such as that associated with light pressure, may interfere with the input from deeper pressure receptors, the response to roughness thus masking the response to more gradual surface changes. The effect of placing material under the fingers may be to reduce this masking, thus allowing the appropriate input to be used in detection.

Ian E. Gordon
Colin Cooper

Department of Psychology,
University of Exeter,
Exeter EX4 4QG, UK

1 Geldard, F. A., *The Human Senses* (Wiley, New York, 1953).
2 Talbot, W. H., Darian-Smith, I., Kornhuber, H. H., and Mountcastle, V. B., *J. Neurophysiol.*, 31, 301–334 (1968).

Gene dosage effect in human trisomy 16

AFTER the discovery of autosomal trisomies in man, the activity of various enzymes in trisomic cells was measured in the belief that proportionality between gene dose and enzyme activity would help in identifying genes carried by trisomic chromosomes[1-5]. It became apparent, however, that the trisomic state could produce changes in enzyme activity unrelated to gene dose, probably by interfering with normal physiological processes[6]. Therefore, verification of the concept that a simple rapport of proportionality between gene dose and concentration of secondary gene product could exist in cells with autosomal trisomy, as it does in diploid cells with gene mutations or in mouse eggs with different numbers of X chromosomes[7], had to await the recent advances in human chromosome mapping. We report here the results of a study of gene dose effects in trisomy 16.

Nine trisomic and eleven control cell strains were used to measure the activity of four enzymes: adenine phosphoribosyl-transferase (APRT, EC 2.4.2.7), which is coded by a gene on chromosome 16 (ref. 8); hypoxanthine–guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8) and glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), which are X-linked; adenosine kinase (AK, EC 2.7.1.20), which is coded by an autosomal gene asyntenic with the APRT locus[9]. Information

Table 1 Origin and chromosome complement of cells analysed

Identification number	Tissue of origin	Gestational age*	Foetal size and development	Chromosome complement
RF3112	Amnion	11	6 mm (SD)	47, XY, +16
RF3138	Amnion	16	9 mm	46, XX
RF3308	Amnion	10	3 mm	47, XY, +16
RF3326	Ovary	17	18 cm	46, XX
RF3391	Amnion	17	IES	47, XX, +16
RF3500	Chorion	10	SD	47, XY, +16
RF3592	Amnion	1	4 mm (D)	47, XY, +16
RF3613	Amnion	1	RS + CS	46, XY
RF3633	Testis	20	18 cm	46, XY
RF3663	Amnion	1	2 mm (SD)	47, XX, +16
RF3679	Amnion	16	RES	47, XX, +16
RF3789	Amnion	11	2 mm (SD)	47, XY, +16
RF3802	Amnion	11	2 mm (SD)	46, XX
RF3817	Amnion	11	9 cm	46, XX
RF3877	Amnion	10	1 cm (SD)	46, XX
RF3905	Amnion	11	RES	46, XX
RF3914	Amnion	11	RS + CS	46, XX
RF3958	Amnion	12	IES	47, XY, +16
H9172	Skin	Adult	Adult	46, XY
H10322	Skin	Adult	Adult	46, XY

* Weeks after last menstruation.
IES, Intact empty sac; RS, ruptured sac; CS, cord stump; RES, ruptured empty sac; SD, severely disorganised; D, disorganised.

Cultures were set up by the plasma clot technique and grown in TC199 supplemented with 1% pooled human serum, 2% chick embryo extract and antibiotics (100 IU penicillin G and 100 μg streptomycin ml⁻¹). Stock cultures were kept in liquid nitrogen and recovered for the purpose of this study. 4×10^6 cells of each strain were collected by treatment with 0.075% solution trypsin in phosphate buffered saline (PBS), washed three times in PBS and lysed by freeze-thawing. APRT, HGPRT and AK activities were measured by the procedures of Long *et al.*[9], G6PD by the method of Giblett[10] and protein by that of Lowry *et al.*[11]. ¹⁴C-hypoxanthine, ¹⁴C-adenosine and ¹⁴C-adenine were obtained from the Radiochemical Centre, Amersham and ATP, TTP, phosphoribosyl pyrophosphate, NADP and glucose-6-phosphate from Sigma.

on the specimens used in this study is summarised in Table 1.

Assays showing a linear relationship between enzyme activity and extract dilution were used for analysis (Fig. 1).

Enzyme assays (Table 2) showed a 69% increase in APRT activity in trisomic cells. Such an estimate has wide confidence limits and does not differ significantly from the 50% increase expected on the hypothesis of direct and linear proportionality between gene dose and enzyme activity. In contrast, HGPRT, AK and G6PD activities do not differ significantly in trisomic and control cells.

As HGPRT and G6PD are X-linked[12,13], we tested whether their activities are influenced by the number of X chromosomes present in the cells—either directly by comparing the levels of

Fig. 1 Graphs showing relationship between cell extract dilution and enzyme activity in a typical experiment. Trisomic (●) and control (○) cell strains were recovered in pairs. When both strains had reached confluency the cells were collected and assayed together to minimise variations caused by the phase of cell growth and the conditions of the assays. Aliquots from the same cell extract were used to assay all the four enzymes and all but G6PD were tested concurrently and in duplicate on three serial dilutions of the cell extract. *a*, HGPRT; *b*, APRT; *c*, AK.
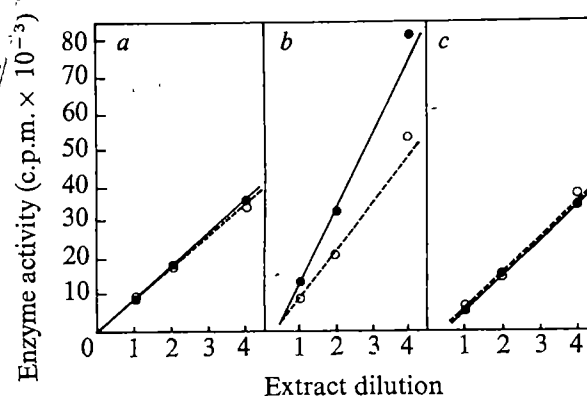
Table 2   APRT, HGPRT, AK and G6PD specific activity in trisomic and control cells

Experimental pair		APRT*		HGPRT†		AK†		G6PD‡	
Trisomic	Control	Trisomic	Control	Trisomic	Control	Trisomic	Control	Trisomic	Control
RF3391	RF3326	210	122	51	50	—	—	—	—
RF3391	RF3138	150	97	45	48	29	20	0 287	0.286
RF3679	RF3138	151	99	47	50	34 5	20	0.287	0.263
RF3663	RF3138	167	108	43.5	53	20	20	0.345	0.286
RF3500	RF3633	148	72	58	54	26.5	20.5	0.363	0.373
RF3500	RF3613	—	—	—	—	—	—	0.230	0.173
RF3500	H 9172	379	197	47	39	—	—	—	—
RF3500	H10322	242	45	55	40	—	—	—	—
RF3592	RF3633	87	24	38	30	27	24	0.289	0.311
RF3789	RF3633	162	76	39	56	25	20.5	0.251	0 293
RF3391	RF3633	109	76	40	56	29	20.5	—	—
RF3679	RF3633	149	76	44	56	34.5	20.5	—	—
RF3663	RF3633	170	77	46	54	20	20.5	—	—
RF3500	RF3817	53	128	32	65	53	116	0.168	0.182
RF3500	RF3138	133	108	54	53	26.5	20	—	—
RF3789	RF3138	162	108	39	53	25	20	—	—
RF3789	RF3914	109	84	42	57	56	76	0.149	0.183
RF3308	RF3905	45	36	54	57	25	28	0.312	0.283
RF3958	RF3877	24	38	—	49	38	49	0.237	0.277
RF3112	RF3802	73	40	40	30	—	43	0.061	0.096
Mean		143.3	84.8	45.2	50.0	31.2	33.0	0.248	0.250
Difference		58.5§		4.8		1.8		0.002	
s.e.		13.9		2.8		4.9		0 010	
Patient/control ratio		1.69		0.90		0.95		0.99	

* AMP nmol per h per mg protein.
† IMP nmol per h per mg protein.
‡ NADP μmol per min per mg protein.
§ Significant at 0.001 level.

enzyme activity in male and female trisomic and control strains or indirectly by comparing the differences in enzyme activities of controls and trisomic cells in pairs of equal sex and pairs formed by a male trisomic and a female control (last seven in Table 2). These tests indicate that the activities of these two enzymes do not differ significantly in male and female control or trisomic cell strains (Table 3).

Past experience suggests caution in the interpretation of increased enzyme activity in trisomic cells. Note that changes in enzyme activity unrelated to gene dose have usually been observed in blood cells. The latter are in a terminal state of differentiation and are usually tested immediately after their withdrawal from the abnormal environment of the trisomic subject. In this study, trisomic and control cells were grown for some time in vitro in identical conditions and analysed in the same growth phase.

Because changes in enzyme activity for physiological rather than strictly genetical reasons may similarly affect enzymes of related metabolic pathways, APRT activity was compared with that of the metabolically related enzymes, HGPRT and AK. In fact the ratio of HGPRT to APRT activity has already been profitably and extensively used in the measurement of gene dose effects in euploid cells—namely, the detection of heterozygotes for mutations at the HGPRT locus[14-16]. The genes coding for the enzymes considered in this work are well expressed in interspecific somatic cell hybrids and seem, therefore, rather insensitive to abnormal genetic situations. In view of these considerations we believe that our observed increases in APRT activity in cells with trisomy 16 is the direct expression of gene dose and is not secondary to a change in cell physiology. Our findings, therefore, provide some evidence for a linear and directly proportional relationship between gene dose and enzyme activity.

A dosage effect for the two loci assigned to chromosome 21 has also been reported in Down's syndrome. Sinet et al.[17] have observed a 40% increase in the activity of the cytoplasmic form of superoxide dismutase (SOD-1) in erythrocytes of patients with trisomy 21. Unfortunately, however, they have not studied the activity of other enzymes or tested SOD-1 in other types of cells. The value of their observation is, therefore, greatly

reduced by the fact that erythrocytes in Down's syndrome have repeatedly shown increases in the activity of enzymes not coded by chromosome 21. Tan et al.[18] have studied the relationship between gene dose and the production of antiviral protein (AVP) in trisomy 21 by measuring the antiviral protection afforded by this protein to trisomic cells. AVP activity is induced by interferon and cells with trisomy 21 have been shown to be three to seven times more responsive to such induction than cells with trisomy 13 or 18, or normal chromosome complement. It is at present unclear whether the nonlinear relationship observed between AVP gene dose and antiviral protection reflects the effect of gene dose on protein synthesis or that of AVP concentration on viral infection. The answer to this dilemma and comparisons between the behaviour of inducible and constitutive enzymes in cells with abnormal karyotypes may help us to understand, in biochemical terms, the phenotypic effect of chromosome imbalance.

Table 3   Sex differences in HGPRT and G6PD activity in trisomic and control cells

	HGPRT activity		G6PD activity	
	Difference	Probability	Difference	Probability
Control cells (♂ against ♀)	−4.3	>0.5	0.063	>0.1
Trisomic cells (♂ against ♀)	0.68	>0 5	−0.077	>0.1
Pairs of equal sex against pairs of unequal sex*	6.2	>0.2	0.028	>0.1

* Only pairs of unequal sex comprising a male trisomic and a female control are considered.

Our HGPRT and G6PD assays seem to support the evidence for dosage compensation of X-linked genes in man by suggesting that compensation takes place even in 8–14-week-old zygotes, whose development has been severely impaired by trisomy 16.

We thank Dr E. Alberman, Mr M. R. Creasy and Mr J. A. Crolla for foetal cell strains, and Professor P. E. Polani for

encouragement and advice. This work was supported by the Spastics Society and by an anonymous gift.

B. MARIMO

F. GIANNELLI

Paediatric Research Unit,
Guy's Hospital Medical School,
London SE1 9RT, UK

1 Alter, A. A., Lee, S. L., Pourfar, M., and Dobkin, G., *J. clin. Invest.*, **41**, 1341 (1962).
2 Alter, A. A., Lee, S. L., Pourfar, M., and Dobkin, G., *Blood*, **22**, 165 (1963).
3 Trubowitz, S., Kirman, D., and Masek, B., *Lancet*, ii, 486 (1962).
4 Brandt, N. J., Frøland, A., Mikkelsen, M., Nielsen, A., and Tolstrup, N., *Lancet*, ii, 700 (1963).
5 Krone, W., Wolf, U., Goedde, H. W., and Baitsch, H., *Lancet*, ii, 590 (1964).
6 Mellman, W. J., Oski, F. A., Tedesco, T. A., Maciera-Coelho, A., and Harris, H., *Lancet*, ii, 674 (1964).
7 Epstein, C. J., *Science*, **163**, 1078 (1969).
8 Tischfield, J. A., Creagan, R. P., and Ruddle, F. H., *Cytogenet. Cell Genet.*, **13**, 167 (1974).
9 Long, C., Chan, T., Levytska, V., Kusano, T., and Green, H., *Biochem. Genet.*, **9**, 283 (1973).
10 Giblett, E. R., *Genetic Markers in Human Blood*, 466 (Blackwell, Oxford and Edinburgh, 1969).
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. biol. Chem.*, **193**, 265 (1951).
12 Tarlov, A. R., Brewer, G. J., Carson, P. E., and Alving, A. S., *Archs intern. Med.*, **109**, 209 (1962).
13 Nyhan, W. L., Pesek, J., Sweetman, L., Carpenter, D. G., and Carter, C. H., *Pediat. Res.*, **1**, 5 (1967).
14 Gartler, S. M., Scott, R. C., Goldstein, J. L., Campbell, B., and Sparkes, R., *Science*, **172**, 572 (1971).
15 Silvers, D. N., Cox, R. P., Balis, E., and Dancis, J., *New Engl. J. Med.*, **286**, 390 (1972).
16 de Bruyn, C. H. M. M., Oei, T. L., and ter Haar, B. G. A., *Clin. Genet.*, **5**, 449 (1974).
17 Sinet, P-M., Allard, D., Lejeune, J., and Jerome, H., *C. r. hebd. Séanc. Acad. Sci. Paris*, **D278**, 3267 (1974).
18 Tan, Y. H., Schneider, E. L., Tischfield, J., Epstein, C. J., and Ruddle, F. H., *Science*, **186**, 61 (1974).

Why didn't Gregor Mendel find linkage?

IT is quite often said that Mendel was very fortunate not to run into the complication of linkage during his experiments. He used seven genes and the pea has only seven chromosomes. Some have said that had he taken just one more, he would have had problems. This, however, is a gross oversimplification. The actual situation, most probably, is shown in Table 1. This shows that Mendel worked with three genes in chromosome 4, two genes in chromosome 1, and one gene in each of chromosome 5 and 7. It seems at first glance that, out of the 21 dihybrid combinations Mendel theoretically could have studied, no less than four (that is, *a–i*, *v–fa*, *v–le*, *fa–le*) ought to have resulted in linkages. As found, however, in hundreds of crosses and shown by the genetic map of the pea[1], *a* and *i* in chromosome 1 are so distantly located on the chromosome that no linkage is normally detected. The same is true for *v* or *le* on the one hand, and *fa* on the other, in chromosome 4. This leaves *v–le*, which ought to have shown linkage.

Mendel, however, seems not to have published this particular combination and thus, presumably, never made the appropriate

Table 1 Relationship between modern genetic terminology and character pairs used by Mendel

Character pair used by Mendel	Alleles in modern terminology	Located in chromosome
Seed colour, yellow–green	*I–i*	1
Seed coat and flowers, coloured–white	*A–a*	1
Mature pods, smooth expanded–wrinkled indented	*V–v*	4
Inflorescences, from leaf axils–umbellate in top of plant	*Fa–fa*	4
Plant height, >1m–around 0.5 m	*Le–le*	4
Unripe pods, green–yellow	*Gp–gp*	5
Mature seeds, smooth–wrinkled	*R–r*	7

cross to obtain both genes segregating simultaneously. It is therefore not so astonishing that Mendel did not run into the complication of linkage, although he did not avoid it by choosing one gene from each chromosome.

STIG BLIXT

Weibullsholm Plant Breeding Institute,
Landskrona, Sweden
and
Centro Energia Nucleare na Agricultura,
Piracicaba, SP, Brazil

1 Blixt, S., in *Handbook of Genetics*, 2 (edit. by King, R. C.), (Plenum, New York, 1974).

Defect of macrophage function in the antibody response to sheep erythrocytes in systemic *Mycobacterium lepraemurium* infection

THE need for macrophages for optimal antibody responses, both *in vivo* and *in vitro*, to certain antigens is now established[1,2] and this paper describes experiments which have used this requirement to demonstrate a deficiency of macrophage function in mice experimentally infected with the rodent leprosy bacillus, *Mycobacterium lepraemurium*. This organism is an obligate intracellular dweller and is found particularly inside cells of the macrophage series although in terminal infection other cell types may be invaded. Systemically infected mice characteristically show macrophages overloaded with bacilli, ever increasing numbers of granulomata and increasing spleno- and hepatomegaly[3]. There is an increase in the phagocytic activity of the spleen and liver at an early stage of infection (I.N.B and V S.S., in preparation) indicative of alterations in macrophage function and we report here experiments which show that the *in vivo* and *in vitro* antibody response to sheep erythrocytes (SRBC) is depressed at later stages of infection. The results indicate a defect of macrophage function.

Young adult CBA female mice were injected intravenously with 10^9 *M. lepraemurium* freshly isolated from the heavily infected livers of mice infected 4–6 months previously (I.N.B. and H. N. Krenzien, in preparation). At 11–15 weeks after infection, when the present experiments were carried out, the mice appeared clinically healthy but at autopsy showed gross enlargement of liver and spleen. Ziehl-Neelsen stain revealed the presence of numerous mycobacteria in macrophages of various tissues, particularly liver, spleen and bone marrow. Uninfected mice of the same age and sex were used as controls The *in vivo* antibody response was measured in mice injected intravenously with either 5×10^6 or 10^9 SRBC. Four days later, the number of antibody producing cells (PFC) present in the spleen of each mouse was determined using the method of Cunningham[4]. The *in vitro* response of spleen cells cultured for 4 d according to the method of Marbrook[5] was measured in the same way.

The results given in Table 1 show that the antibody response *in vivo* was reduced 11 weeks after infection and even more so at 14 weeks. This depression was more pronounced in animals injected with the smaller number of SRBC. It seemed greater when the results were expressed as PFC per 10^9 spleen cells because there was a 4–5-fold increase in the cellularity of the spleens from infected compared with normal mice. Serum antibody levels were also reduced in infected mice (results not shown). Spleen cells from infected animals cultured *in vitro* in the presence of SRBC failed to generate the expected number of PFC, particularly at 14 weeks.

Table 1 Antibody response of mice, or spleen cells from mice, infected with *M. lepraemurium*

Parameter of response	Dose of SRBC	Control	Weeks after infection	
			11	14
In vivo				
$\log_{10}$ PFC per spleen	5×10^6	5.85 ± 0.02	5.33 ± 0.06	4.87 ± 0.16
	10^9	5.97 ± 0.04	5.76 ± 0.07	5.28 ± 0.03
PFC per spleen, geometric	5×10^6	703	214	75
mean ($\times 10^{-3}$)	10^9	937	570	189
PFC per 10^6 spleen	5×10^6	$6,051 \pm 391$	605 ± 100	204 ± 83
cells	10^9	$4,899 \pm 452$	$1,250 \pm 154$	446 ± 21
In vitro				
PFC per culture	—	$2,086 \pm 142$	$1,246 \pm 57$	290 ± 39

Spleen cells were cultured in RPMI 1640 medium containing HEPES, bicarbonate and 5% foetal calf serum. The cultures contained 20×10^6 spleen cells and 2×10^6 SRBC. The values shown are means $\pm$ s.e. of four mice in each group or quadruplicate cultures of spleen cells pooled from two or three mice. The significance of difference between control and experimental groups, calculated using the two-tailed Student/Welch's t-test[6], was at the level of $P < 0.005$ or higher.

Further studies were carried out *in vitro* to obtain information about the functional status of macrophages and lymphoid cells in infected spleens. Cells from infected and control mice were fractionated into adherent (macrophage) and non-adherent (lymphocyte) populations and were then cultured in various combinations in the Marbrook system. The results are shown in Table 2. Cultures containing only non-adherent cells produced small numbers of PFC and no PFC were detected in cultures of peritoneal cells or adherent spleen cells (not shown in the table). When non-adherent cells from the spleens of normal mice were supplemented with normal peritoneal cells or adherent

spleens were supplemented with either normal peritoneal cells or normal spleen adherent cells (experiments 2 and 3).

The results presented here indicate, in contrast to another report[9], that the antibody response to SRBC is depressed in mice systemically infected with *M. lepraemurium*. Furthermore, the defect was associated particularly with the macrophages of infected animals and not with their lymphocytes at the stages of infection studied. Our experiments do not indicate a direct contact effect by heavily infected macrophages or the release of a suppressive factor. The nature of the defect has yet to be established. Two possibilities are being considered in our immediate experi-

Table 2 The *in vitro* antibody response of spleen cells from mice infected with *M. lepraemurium*

Experiment	Cells cultured	PFC per culture		
			Weeks after infection	
		Control	11	14–15
1	Spleen unfractionated	$2,140 \pm 82$	960 ± 57	620 ± 22
2	Spleen non-adherent + normal PC	$2,140 \pm 85$	$1,790 \pm 78$	$1,466 \pm 33$
3	Spleen non-adherent + normal spleen adherent	$1,886 \pm 41$	$1,800 \pm 26$	$1,706 \pm 63$
4	Spleen unfractionated + normal PC	$2,600 \pm 10$	$1,716 \pm 51$	$1,510 \pm 59$
5	Spleen non-adherent	190 ± 12	96 ± 46	80 ± 34
6	Normal spleen non-adherent + infected spleen adherent	—	$1,192 \pm 64$	610 ± 13

Cultures of unfractionated spleen cells contained 20×10^6 cells ml^{-1}. Non-adherent cells were obtained by incubating spleen cells (20×10^6 ml^{-1}) twice for 30 min at 37 °C on glass and the free cells in the suspension were then gently agitated and poured off and 1 ml put into each culture vessel. The adherent spleen cells were removed from the glass surface by means of a rubber policeman before which the surface was vigorously washed with fresh medium. Peritoneal cells (PC) were obtained by washing the peritoneal cavity of untreated mice. Cultures were supplemented with macrophages by adding 2×10^6–3×10^6 viable adherent spleen cells or 4×10^6 peritoneal cells. All cultures contained 2×10^6 SRBC. Values given are means $\pm$ s.e. of quadruplicate cultures.

spleen cells (experiments 2 and 3), the PFC response was similar to that found in cultures of unfractionated normal spleen cells. This result confirms earlier findings[7,8] on the requirement for macrophages in the primary response to SRBC *in vitro*. On the other hand, when adherent cells from the spleens of mice infected 11 or 14–15 weeks before were used to supplement non-adherent normal mouse spleen cells (experiment 6) the number of PFC found in the cultures was similar to that found in cultures of unfractionated spleen from infected animals (experiment 1). This result suggested that macrophages from infected mouse spleens were defective in some respect or, alternatively, that they had an inhibitory effect on the function of lymphocytes. These possibilities were tested by adding normal mouse peritoneal cells to cultures of unfractionated spleen cells from normal or infected animals (experiment 4). Under these conditions, the PFC response of the cultures prepared from infected animals was substantially improved (compare experiments 1 and 4) indicating that the presence of infected macrophages did not greatly interfere with the PFC response provided that adequate numbers of normal macrophages were present.

Further evidence for the essentially normal function of the lymphoid cells of infected mice was obtained from experiments where non-adherent cells from infected mouse

ments. First, that overloading of macrophages with mycobacteria may interfere with their ability to ingest, process and present other antigens in a normal manner[10], and second, a possibility associated with the massive antibody production against *M. lepraemurium* antigens during infection, that antigen–antibody complexes are formed and become bound to the surface of macrophages, thus blocking the sites for binding of the factor(s) produced by T lymphocytes which may be required for antibody production to SRBC. Whether or not macrophage defects of a similar nature occur in diseases of man where macrophage involvement is extensive, for example, leprosy, leishmania and malaria, requires further study. If they do, they could account, at least in part, for the immunosuppressed state often associated with such infections.

SUSAN R. WATSON
V. S. SLJIVIĆ
I. N. BROWN

Departments of Immunology and Bacteriology,
St Mary's Hospital Medical School,
London W2 1PG, UK

[1] Feldman, M., and Globerson, A., in *Immunogenicity* (edit. by Borek F.) 273–301 (North-Holland, Amsterdam, 1972).

2 Saito, K., *Recent Advances in RES Res.*, 12, 1–17 (1972).
3 Nishimura, S., *et al.*, in *Mykobakterien und mykobakterielle Krankheiten*, (edit. by Meissner, G., and Schmiedel, A.), 9, 289–416 (Fisher Verlag, Jena, 1967).
4 Cunningham, A. J., and Szenberg, A., *Immunology*, 14, 599–600 (1968).
5 Marbrook, J. *Lancet*, ii, 1279–1281 (1967).
6 Cochran, W. G., and Cox, G. M., *Experimental Design*, 100 (Wiley, New York, 1957).
7 Mosier, D. E., *Science*, 158, 1573–1575 (1967).
8 Sjoberg, O., Andersson, J., and Möller, G., *Eur. J. Immun.*, 2, 123–126 (1972).
9 Ptak, W., Gaugas, J. M., Rees, R.J.W. and Allison, A. C., *Clin. Exp. Immun.*, 6, 117–124 (1970).
10 Kölsch, E., in *Mononuclear Phagocytes* (edit. by van Furth, R.), 548–560 (Blackwell, Oxford, 1970).

Promotion of cell fusion by divalent cation ionophores

THE importance of cytoplasmic calcium has recently been emphasised by demonstrations that the ionophores X537A and A23187, which are known to facilitate the movement of Ca^{2+} through membranes[1,2], trigger secretion in the presence of extracellular calcium ions[3,4]. We now report observations on the effects of these two cation ionophores on hen erythrocytes at physiological *p*H, which raise the possibility that the phenomenon of cell fusion may also be mediated by an increased concentration of cytoplasmic Ca^{2+}.

A suspension of hen erythrocytes (about 6×10^8 cells ml^{-1}) in buffered solution (A), (NaCl 116 mM, $CaCl_2$ 1 mM, Me_2AsO_2Na 10 mM, glucose 1 g l^{-1}, penicillin G 200,000 units l^{-1}, streptomycin sulphate 100 mg l^{-1}; *p*H 7.4) was prepared[5]. After centrifugation, 1 ml of the packed cells was incubated with 250 units of neuraminidase solution (*Vibrio cholerae*) (0.5 ml; Behringwerke AG) for 30 min at 37 °C. The treated cells were then washed twice with the buffered salt solution (10 ml), diluted to give a cell suspension of $\sim 6 \times 10^8$ cells ml^{-1}, and kept at 4 °C until used later on the day of preparation.

Suspensions of neuraminidase-treated cells ($\sim 2 \times 10^8$ cells ml^{-1}) in buffered salt solution (A) containing dextran (80 mg ml^{-1}; MW 82,000; Sigma Chemical Co.), were incubated at 37 °C with either X537A or A23187 (6.6 µg ml^{-1}). X537A and A23187 were given by Roche Products and Eli Lilly respectively. The ionophores were dissolved in ethanol (1 mg ml^{-1}). Small aliquots of cell suspension were removed at intervals for microscopy. In control experiments, neuraminidase-treated cells were incubated with ethanol (6.6 µl ml^{-1}) in the dextran-containing medium, free from ionophore.

After treatment with neuraminidase, the erythrocytes aggregated into large clumps on incubation at 37 °C with dextran, and the outlines of individual cells in these clumps could not easily be distinguished (Fig. 1*a*). A similar dextran-induced aggregation has been reported to occur with neuraminidase-treated human erythrocytes[6]. When ionophore was also present, individual cells in the clumps became rounded and swollen within 15 min. The nuclei were clearly visible in these preparations and limited cell fusion may have occurred after 3 h (Fig. 1*b*). A further incubation of the cell suspensions at 47 °C for 10 min resulted in extensive fusion (Fig. 1*d*). Essentially similar results were obtained with both ionophores. Without ionophore, the neuraminidase-treated, dextran-aggregated cells were apparently unaffected after 30 min further incubation at 47 °C (Fig. 1*e*). Electron microscopy confirmed cell fusion in the cells treated with ionophore (Fig. 1*c*), but no fusion was seen in its absence. Only a limited degree of cell fusion occurred, even at 47 °C, when the concentrations of ionophores were reduced tenfold (0.66 µg ml^{-1}).

A first requirement for cell fusion is cell contact. In the presence of lysolecithin hen erythrocytes aggregate and fuse at *p*H 5.6 (ref. 7). With fusogenic lipids, for example glyceryl mono-oleate, fusion at *p*H 5.6 is facilitated by dextran which increases the aggregation of hen erythrocytes treated with the fusogen[5]. Following neuraminidase action, the cells are more rapidly fused even at *p*H 7.4 by glyceryl mono-oleate, in the presence of dextran[8]. Divalent cation ionophores seem to have little aggregating action and, for fusion to occur rapidly, it seems to be necessary to treat the cells with neuraminidase and then to aggregate them with dextran. Cell fusion occurred, however, in the presence of 2 mM Ca^{2+}, even at 37 °C, with hen erythrocytes that were not pretreated with neuraminidase but were incubated in dextran-containing media for 5 h with A23187 or 18–22 h with X537A.

Membrane fluidity is also important in cell fusion[8,9], and changes in membrane structure resulting from treatment with X537A and A23187 that lead to cell fusion are markedly accelerated by raising the temperature to 47 °C. (Thermally induced cell fusion at 48–50 °C and *p*H 5.6 in the absence of ionophore, which was reported earlier[8], occurs only on a heated, microscope stage and not with cells in free suspension.) Alternatively benzyl alcohol (30 mM), which increases membrane fluidity[10] but is not fusogenic at this concentration[11], may be used instead of heat to aid the induction of cell fusion by ionophore. Mechanical tapping of a microscope slide
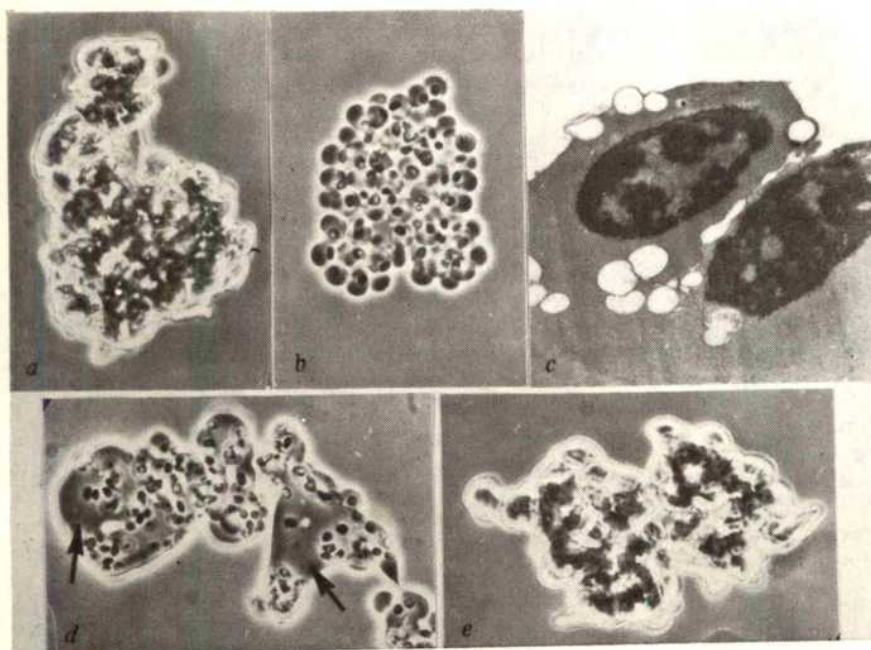
Fig. 1 *a, b, d* and *e,* Light micrographs (phase contrast, ×300) of neuraminidase-treated hen erythrocytes at *p*H 7.4 prepared as described in the text. *a,* A clump of aggregated cells formed on incubation at 37 °C for 5.75 h with dextran (80 mg ml^{-1}) and ethanol (6.6 µl ml^{-1}). *b,* Swollen, aggregated cells arising on incubation at 37 °C for 3 h with dextran (80 mg ml^{-1}), ethanol (6.6 µl ml^{-1}), and X537A (6.6 µg ml^{-1}). *d,* Cells treated as in *b* followed by incubation at 47 °C for 10 min: multinucleated syncytia are present that contain aggregated nuclei, and areas of nuclei-free cytoplasm (arrows). *e,* Cells treated as in *b,* but without ionophore, followed by incubation at 47 °C for 30 min. *c,* An electron micrograph of a thin section, prepared as described previously[7], of cells that were incubated at 37 °C for 1 h with dextran (80 mg ml^{-1}), ethanol (6.6 µl ml^{-1}), and A23187 (6.6 µg ml^{-1}), followed by 45 min at 47 °C, showing two nuclei in a common cytoplasm: vacuolation of the plasma membrane is also apparent (×8,250).

bearing a preparation like that shown in Fig. 1b will also trigger some of these cells to fuse.

Light microscope experiments provided no evidence that either valinomycin (6.6 μg ml^{-1}), with or without 2,4-dinitrophenol (0.1 mM), or 2,4-dinitrophenol alone caused the erythrocytes to fuse. Increasing the concentration of Ca^{2+} from 1 to 3 mM allowed the changes, including cell fusion, caused by X537A and A23187 to occur more rapidly, but was without effect on cells treated with valinomycin or valinomycin and DNP. The actions of X537A and A23187 with 1 mM Ca^{2+} were inhibited by EGTA and EDTA (2 mM); inhibition by the chelating agents was overcome by excess Ca^{2+} (final concentration 3 mM). It may therefore be concluded—as with secretory systems[3,4]—that the observed fusogenic action of these two divalent ionophores is mediated by entry of Ca^{2+} into the cells.

Ca^{2+} probably facilitates cellular aggregation in cell fusion. On the basis of our experiments it is also conceivable that Ca^{2+} may mediate cell fusion, occurring either naturally or in response to fusogenic agents[5], by interacting with the cytoplasmic side of the plasma membrane following an initial increase in membrane permeability to exogenous Ca^{2+}. This would apparently be contrary to the views of Poste and Allison[12] who have postulated that membranes can exist in two states, (1) the normal Ca^{2+}-associated state; and (2) the "fusion susceptible" state in which Ca^{2+} has been displaced from the membrane.

Cell swelling, which accompanies fusion induced by ionophores and lipid fusogens[5], may be a result of inhibition by cytoplasmic Ca^{2+} of membrane-bound Na$^+$/K$^+$-dependent ATPase activity, with a resultant loss of cytoplasmic potassium[13], and the entry of external water as postulated for the Ca^{2+}-dependence of the acrosome reaction[14]. Ca^{2+} may also be expected to aggregate negatively charged spectrin molecules on the cytoplasmic surface: this will, in turn aggregate integral membrane proteins[15]. Furthermore the binding of Ca^{2+} to phosphatidylserine molecules in a membrane yields solid aggregates that allow other phospholipids to form fluid clusters[16]. If this should occur at the cytoplasmic surface of the erythrocytes, where most of the phosphatidylserine is located[17], it would probably also lead to an aggregation of intramembranous proteins[18]. Aggregation of membrane proteins[12,19] and the clustering of lipid molecules[19] are thought to be important in membrane fusion. These phenomena may therefore all participate in cell fusion induced by an increase in the concentration of cytoplasmic Ca^{2+}. Some of these suggestions may also be relevant to the Ca^{2+}-mediated fusion of membranes that occurs in secretory processes[20,21].

Q. F. AHKONG
W. TAMPION
J. A. LUCY

Department of Biochemistry and Chemistry,
Royal Free Hospital School of Medicine,
University of London,
8 Hunter Street,
London WC1N 1BP, UK

1 Reed, P. W., and Lardy, H. A., in *The Role of Membranes in Metabolic Regulation* (edit. by Mehlman, M. A., and Hanson, R. W.), 111–131 (Academic Press, New York, 1972).
2 Pressman, B. C., *Fedn Proc.*, 32, 1698–1701 (1973).
3 Cochrane, D. E., and Douglas, W. W., *Proc. natn. Acad. Sci. U.S.A.*, 71, 408–412 (1974).
4 Foreman, J. C., Mongar, J. C., and Gomperts, B. D., *Nature*, 245, 249–251 (1973).
5 Ahkong, Q. F., Fisher, D., Tampion, W., and Lucy, J. A., *Biochem. J.*, 136, 147–155 (1973).
6 Jan, K.-M., and Chien, S., *J. gen. Physiol.*, 61, 638–654 (1973).
7 Howell, J. I., and Lucy, J. A., *FEBS Lett.*, 4, 147–150 (1969).
8 Ahkong, Q. F., Cramp, F. C., Fisher, D., Howell, J. I., Tampion, W., Verrinder, M., and Lucy, J. A., *Nature new Biol.*, 242, 215–217 (1973).
9 Papahadjopoulos, D., Poste, G., and Schaeffer, B. E., *Biochim. biophys. Acta*, 323, 23–42 (1973).
10 Metcalfe, J. C., Seeman, P., and Burgen, A. S. V., *Molec. Pharmac.*, 4, 87–95 (1968).
11 Fisher, D., in *NATO Symposium on Lipids, Proteins and Receptors* (edit. by Burton, R. M., and Packer, L.), (Bi-Science Publications Division, Webster Groves, Missouri, in the press).
12 Poste, G., and Allison, A. C., *Biochim. biophys. Acta*, 300, 421–465 (1973).
13 Dunn, M. J., *Biochim. biophys. Acta*, 352, 97–116 (1974).
14 Yanagimachi, R., and Usui, N., *Expl Cell Res.*, 89, 161–174 (1974).
15 Nicolson, G. L., and Painter, R. G., *J. Cell Biol.*, 59, 395–406 (1973).
16 Ohnishi, S-I., and Ito, T., *Biochemistry*, 13, 881–887 (1974).
17 Zwaal, R. F. A., Roelofsen, B., and Colley, C. M., *Biochim. biophys. Acta*, 300, 159–182 (1973).
18 Grant, C. W. M., and McConnell, H. M., *Proc. natn. Acad. Sci. U.S.A.*, 71, 4653–4657 (1974).
19 Ahkong, Q. F., Fisher, D., Tampion, W., and Lucy, J. A., *Nature*, 253, 194–195 (1975).
20 Douglas, W. W., *Biochem. Soc. Symp.*, 39, 1–28 (1974).
21 Rubin, P., *Calcium and the Secretory Process* (Plenum, New York, 1974).

Differential formation of desmosomes and hemidesmosomes in epidermal cell cultures treated with retinoic acid

IN various epithelial tissues, desmosomes (D) as well as the hemidesmosomes (HD) are common specialisations of the plasma membrane[1]. Both membrane differentiations are thought to play an important role either in cell–cell adhesion (D) or in the attachment to biological substrata (HD)[2]. *In vitro* formation of D and HD has been observed in embryonic lens cells[3] or organ culture outgrowth of skin[4]. We have now found that in cultured post-embryonic epidermal cells (PEC) both membrane specialisations are formed independently of each other. We show that in cells stimulated to grow, an increased number of attachment devices contacting the substratum is formed. At the dorsal surface of the same cell, however, morphological expression of tissue-specific differentiation is present.

In cultured PEC (ref. 5), nuclear DNA synthesis as well as cellular growth is greatly enhanced by the addition of retinoic acid (RA) to the culture medium. Comparison with other cell systems present in skin indicates tissue specificity[6]. The mitogenic effect of RA was therefore used to study the formation of membrane specifications under different growth conditions.

Figure 1 shows that numerous specialisations of the plasma membrane are present in 3-d-old cultures. HD are exclusively present at the underside of 'basal' cells contacting the plastic substratum. On the other hand, D are located at the upper side of 'basal' cells facing overlying epidermal cells. The morphology of both, HD and D, does not seem to be different from previous *in vivo* descriptions[1,2].

Twice as many HD were present at the undersurface of RA-treated cells by comparison with controls ($P < 0.005$, Fig. 2). Functionally, this greater number of HD present in treated cells should indicate increased adherence to the substratum. Considerably longer exposure times to trypsin are necessary, however, to collect RA-treated cells as compared with untreated controls (E. C. and H. H. W., unpublished).

The number of D at the dorsal site of the 'basal cell' plasma membrane contacting the suprabasal cell did not change with treatment (Fig. 2). Obviously, within the same cell the site-dependent attachment devices are formed independently.

In epithelial cells freshly treated with trypsin, D and HD are absent[3], and are therefore most likely to be newly formed during cellular reaggregation. Also, formation of D depends on the presence of adjacent cells of the same species and of a corresponding maturational age[3]. Our observed distribution of both membrane specialisations follows the *in vivo* pattern of epidermal differentiation.

In contrast to tumour cells, so-called 'normal' cells show strong anchorage dependence when grown *in vitro*[7]. It has been found[8] that changing the substratum (from glass to agarose) under otherwise identical conditions resulted in

decreased adherence of cells, reduced rate of DNA synthesis and increased generation time. It thus seems that in normal cells attachment to the substratum is linked to cellular proliferation.

There is evidence that in epidermal cells such fixation to the substratum is provided by hemidesmosomes. Our observation of a greatly increased number of HD in rapidly multiplying cells supports the concept that these membrane specialisations are positively correlated with cell proliferation. Under *in vivo* conditions strong adherence to the basal

Fig. 2 Number of HD (*a*) and D (*b*) in 3-d-old cultures of PEC treated with 10 µg ml^{-1} RA (hatched bars) and untreated controls (open bars). Epon-embedded specimens were obtained after *in situ* fixation of entire culture plates. Selected areas were excised and cut with a diamond knife. Vertical sections were stained with uranyl acetate and lead citrate, and viewed with a Philips EM300 microscope. Photomicrographs were taken for HD and D counts at magnifications between 8,000 and 26,000. Length of plasma membrane on both sides of the cells was measured using a map roller. HD and D were counted and the results calculated as HD or D per 10 µm surface length + s.e.

lamina would thus be provided for cells engaged in reduplication.

On the other hand, upward movement and tissue-specific differentiation of epidermal cells coincides with the disappearance of hemidesmosomes and increased formation of desmosomes. The basic functional difference between the two attachment devices of the epidermal cell membrane thus seems to be closely linked to the mechanism of epidermal cell renewal.

This work was supported by the Deutsche Forschungsgemeinschaft and SFB 51, Munich.

E. CHRISTOPHERS
H. H. WOLFF

Department of Dermatology,
University of Munich,
Frauenlobstrasse 9,
8 München 2, West Germany

1 Farquhar, M., and Palade, G. E., *J. Cell Biol.*, 17, 375–412 (1963).
2 Odland, G. F., and Reed, T. H., in *Ultrastructure of Normal and Abnormal Skin* (edit. by Zelickson, A. S.), (Lea and Febiger, Philadelphia, 1967).
3 Overton, J., *J. Cell Biol.*, 56, 637–646 (1973).
4 Flaxman, B. A., Lutzner, M. A., and Van Scott, E. J., *J. Cell Biol.*, 36, 406–410 (1968).
5 Christophers, E., *J. invest. Dermat.*, 63, 450–455 (1974).
6 Christophers, E., and Langner, A., *Arch. Derm. Forsch.*, 251, 147–153 (1975).
7 Stoker, M. G. P., O'Neill, C. H., Berryman, S., and Waxman, V., *Int. J. Cancer*, 3, 683–692 (1968).
8 Riley, P. A., *Cell Differentiation*, 3, 233–238 (1974).

Fig. 1 High density epidermal cell culture grown *in vitro* for 3 d (control). Two types of membrane specialisations are formed: D, located at sites in contact with adjacent cells (*a*), and HD, exclusively present at the undersurface of cells contacting the substratum (*b*). Cultures were prepared[5] by gentle treatment with trypsin of adult guinea pig ear skin fragments. Cell suspensions consisting of single cells were seeded at a density of 1.8–2.2 × 10⁶ ml^{-1} in 35 mm Falcon culture dishes and maintained in 5% CO_2–air at 37 °C. *trans*-Retinoic acid (Nordmark-Werke, Uetersen) was dissolved in DMSO and added to the culture medium (McCoy 5a, supplemented with 10% foetal calf serum, penicillin and streptomycin) at a final concentration of 10 µg ml^{-1}. The concentration of DMSO in all cultures, including controls, was 0.1%. *a*, ×27,540; *b*, ×60,000.

Demonstration of intermolecular forces in cell adhesion using a new electrochemical technique

THE problem of cell adhesion has been approached from both chemical[1-3] and physical[4,5] viewpoints. We present here new evidence that a purely physical attraction acts between cells and other surfaces and that electrostatic forces can overcome this attraction. Previous attempts to demonstrate

and interpret such forces[6-8] have largely been frustrated by inadequately defined test surfaces, biological media including complex macromolecular components, and inappropriate assays for cell adhesion.

The unique features of our experimental approach are that the interfacial electrostatic charge of a well defined clean metallic surface can be varied continuously from positive to high negative values, and measured accurately. We have consequently been able to determine the critical electrostatic condition for cell adhesion. Another significant feature of our system is that the very low contamination rate of the test surface can be monitored continually during the course of the experiment.

We have used the relatively new development of solid polarisable electrodes: the test surface was a lead electrode[9] whose high polarisability in contact with electrolyte enables the interface to be charged positively or negatively by means of a battery and potentiometer: the magnitude of the charge being determined accurately from differential capacitance measurements. Since a current density of only a few $\mu A\ cm^{-2}$ flowed across the electrode surface, the experiment was virtually electrostatic. The lead surface was prepared by chemical polishing, after which it is probably molecularly smooth over large areas. Contamination of the electrode by oxygen, the adsorption of ions or organic molecules, could be detected by capacitance changes, providing a criterion for the rejection of poorly prepared electrodes. In practice, high stability over a 6-h period was frequently achieved following exhaustive cleaning and deoxygenation of the entire system.

We examined the adhesion of glutaraldehyde-fixed human red blood cells to the electrode in dilute sodium fluoride solutions of electrochemical purity. Aldehyde fixation, which has little or no effect on cell surface charge[10], was necessary to render cells stable at low ionic strength and to avoid loss of cellular proteins which might otherwise contaminate the electrode. Adhesion was assessed by allowing cells to settle for twenty minutes (settling for a period of 1 h does not affect the results) on to the polarised electrode and then inverting it while under continuous microscopic observation: cells which fell off were scored as non-adhesive. Cell falling was usually completed in 10 min.

In 10 mM NaF all cells stuck to the electrode, even at high negative surface charge. As the concentration was reduced to 1.1 mM, however, a most interesting pattern of behaviour was seen. When the electrode was positively charged ($+1.2 \times 10^3$ e.s.u. cm^{-2}) the negatively charged cells adhered irreversibly—our criterion for irreversibility being that the cells continued to adhere when the negative polarisation was subsequently increased to the maximum value of -4.1×10^4 e.s.u. cm^{-2}, approximately ten times the cell surface charge density. But when cells were allowed to settle on the negatively polarised electrode, within the range -5×10^3 to -3×10^4 e.s.u. cm^{-2}, reversible adhesion occurred. The cells failed to fall off when the electrode was inverted even after 20 min, but within a few minutes of the negative polarisation being increased beyond -3×10^4 e.s.u. cm^{-2} most of the cells were forced off electrostatically and were seen to sediment away from the electrode under the effect of gravity. Cells which adhered to the electrode at different charge densities within the range -5×10^3 to near -3×10^4 e.s.u. cm^{-2} fell off close to the critical charge density of -3×10^4 e.s.u. cm^{-2}.

The explanation for these entirely novel observations can be provided in terms of electrostatic and electrodynamic forces. The physical force theory[5] predicts strong, probably irreversible, adhesion at the limit of molecular approach (primary potential energy minimum) and a weaker reversible adhesion where cell and surface are separated by a finite gap and the forces of attraction and repulsion are equal (secondary potential energy minimum), the two energy minima being separated by an energy barrier.

Preliminary calculations suggest that the distance of closest approach between a red cell and the metal surface may be several hundred Å in the secondary minimum position. We therefore feel that the essential criteria for the duplex adhesive behaviour predicted by the physical force theory have been demonstrated, and it is hard to think of an alternative explanation for the experimental facts. It is anticipated that when the distance of separation between cell and electrode has been measured directly it will be possible to estimate the size of the attractive force from the repulsion and make a quantitative comparison with the theory.

Whether secondary minimum adhesion occurs in physiological conditions remains uncertain. In our experimental system it has been found only at very low ionic strength where electrostatic repulsion can be maximised. But the calculated electrodynamic attraction to metal is large[5], so that lower repulsion coupled with lower attraction may give reversible secondary minimum adhesions in physiological media. Further experiments on cell adhesion at charged oil–water interfaces[11] may answer this question.

Our experiment has demonstrated that attractive forces which presumably include both image forces and electrodynamic (van der Waals') forces, and repulsive electrostatic forces, all of which can act over distances greatly in excess of chemical bond lengths, are responsible for the adhesive behaviour of red blood cells to a defined surface in the most stringently controlled conditions yet used in such work. The very nature of these rigorous controls makes the experiment unavoidably non-physiological; this, however, by no means detracts from the fact that long range interactions have been shown to occur between the complex architecture of protein, lipids and glycoprotein comprising the cell periphery, and the atoms of a solid surface. The conclusion that these forces also operate between cells would seem to be unavoidable.

D. GINGELL
J. A. FORNES*

Department of Biology, as Applied to Medicine,
The Middlesex Hospital Medical School,
London W1P 6DB, UK

*Permanent address: Department de Fisica, Universidad Nacional de La Plata, Box 67, La Plata, Argentina.

1 Cauldwell, C. B., Henkart, P, and Humphreys, T., Biochemistry, 12, 3051–3055 (1973).
2 Roseman, S., Chem Phys. Lipids, 5, 270–297 (1970).
3 Roth, S., Q. Rev Biol., 48, 541–563 (1974).
4 Curtis, A. S. G, The Cell Surface its Molecular Role in Morphogenesis (Academic, New York and London, 1967).
5 Parsegian, V A, and Gingell, D, in Recent Advances in Adhesion (edit. by Lee, L. H), 153–190 (Gordon and Breach, London, 1973).
6 Weiss, L., Expl Cell Res, 53, 603–614 (1968).
7 George, J. N, Weed, R. I., and Reed, C. F., J. Cell Physiol., 77, 51–59 (1971).
8 Maroudas, N. G, J. theor. Biol., 49, 417–424 (1975).
9 Rybalka, K. V., and Leikis, D. I, Elektrokhimiya, 3, 383–386 (1966).
10 Seaman, G V. F., and Cook, G. M. W, in Cell Electrophoresis (edit. by Ambrose, E J.), 48 (Churchill, 1965).
11 Gingell, D., and Todd, I., J. Cell Sci., 18, 227–239 (1975).

Increased spontaneous transmitter release from presynaptic nerve terminal by methylmercuric chloride

IN recent years, several incidences of methylmercury pollution have been documented[1,2], one of the most striking alterations induced by the compound being extensive damage to the nervous system. Experimental mercury poisoning has been produced in animals[3,5], detailed morphological studies of which have shown that acute changes occur initially in the peripheral nerve fibres and thereafter in the central nerve cells[3,4]. Little

is known, however, about functional changes in the nervous system, particularly in the initial stages of poisoning. This report describes one possible target site—the synaptic transmission in the sympathetic ganglia—for methylmercury poisoning. The study is also of interest, as a number of heavy metallic ions such as La^{3+}, Co^{2+} and Mn^{2+} have recently been reported to have profound effects on neuromuscular transmission[6-9].

solution. Table 1 summarises the results together with those from untreated control animals. Values for the resting potentials and amplitudes of the action potentials were within the control ranges. There was no sign of impairment of impulse transmission from the preganglionic nerve to ganglion cells (Fig. 1a and b). Amplitudes of the spontaneous miniature excitatory postsynaptic potentials (MEPSPs) were also unaffected, but frequencies were increased significantly (Fig. 1c and d), their

Table 1 Electrical activities of the sympathetic ganglion cells before (control) and after a single intraperitoneal injection of CH_3HgCl (1 mg per animal)

	Resting potential (mV)	Action potential[†] (mV)	mEPSP Amplitude (mV)	mEPSP Frequency (s^{-1})	Conduction velocity[‡] $(m\ s^{-1})$
Control	51± 5 (43)	62± 8 (43)	1.4±0.6 (25)	0.11 ±0.09 (25)	1.5±0.6 (25)
After 30 min	54± 7 (14)	65±11 (14)	1.3±0.3 (12)	0.56*±1.01 (12)	1.8±0.6 (14)
After 1 d	50± 5 (10)	60± 9 (10)	1.6±0.5 (10)	0.28*±0.31 (10)	1.4±0.5 (11)
After 5 d	52± 4 (13)	61± 6 (13)	1.6±0.5 (12)	0.26*±0.27 (12)	1.3±0.4 (12)
After 12 d	51± 3 (27)	60± 6 (23)	1.6±0.4 (22)	0.28 ±0.56 (22)	1.3±0.6 (19)
After 30 d	52± 5 (9)	60± 7 (8)	1.6±0.4 (13)	0.10 ±0.09 (13)	1.6±0.9 (14)

Values are means±s.d. Numbers in parentheses indicate number of cells analysed.
*Statistical significance in the difference from control ($P < 0.05$).
†Amplitudes of the action potentials evoked by the preganglionic nerve stimulation.
‡Velocities of the most rapidly conducting impulses in the preganglionic nerve, approximated by an estimation from latency of the first EPSP and distances between stimulating and recording electrodes.

Superior cervical ganglia with the preganglionic nerve fibres isolated from guinea pigs (160–240 g) were used. Preparations were mounted in a constant temperature bath (34–36 °C) perfused with a modified Krebs solution, as described previously[10]. A single microelectrode filled with 0.6 M K_2SO_4 was used to record intracellularly the membrane potential of individual ganglion cells. The preganglionic nerve fibres were stimulated with twin platinum electrodes to evoke orthodromic responses in ganglion cells.

In the first series of experiments, each guinea pig was given 1 mg methylmercuric chloride (CH_3HgCl) intraperitoneally in aqueous solution. Inspection of the animals at any time after injection revealed no neurological symptoms of the mercury poisoning, such as an ataxic gait or a crossing phenomenon in the hind limbs, reported to occur in rats when total doses of the administration amounted to more than 10 mg (ref. 3). At 30 min, 1, 5, 12 and 30 d after injection, ganglia were isolated and measurements of the electrical activities made in normal

Fig. 1 a, Typical record of action potential and EPSPs in response to three successive stimulations of preganglionic nerve, from an intact guinea pig. b, As a, but killed 30 min after intraperitoneal injection of 1 mg CH_3HgCl. c, Typical record (continuous from top to bottom) of MEPSP from intact guinea pig. d, As c, but 30 min after injection. Note increase in frequency of MEPSP in record d. Calibration, 10 mV and 10 ms for records a and b; 5 mV and 0.1 s for records c and d. Records retouched.

mean values within 5 d of injection being 240–500% greater than control. After 12 d, there was a similar increase in the mean value, but no statistical significance. This is probably caused by a large variability in values for individual ganglion cells. After 30 d, the increased frequency returned to the control level.

In patients suffering from effects of Niigata methylmercury pollution in Japan, Kanbayashi et al.[11] have found a significantly reduced value for the velocity of the most rapidly conducted impulse in the motor nerves. On the other hand, Von Burg and Rustam[12] have found negative results in the patients of an outbreak in Iraq. In agreement with the latter, our study shows no significant change in the velocity of the preganglionic nerve (Table 1).

Thus, the first series of experiments shows that one of the earliest manifestations of CH_3HgCl poisoning with small doses could be detectable as an increase in spontaneous transmitter release from the preganglionic nerve terminal, and that the animal recuperates within a relatively short time after such poisoning.

In the second series of experiments, isolated ganglia from intact animals were exposed continuously to a solution of CH_3HgCl (0.04 mM; 10^{-5} g ml^{-1}). Fig. 2a, recorded in normal solution, shows a typical MEPSP occurring at a frequency of 0.13 s^{-1}. Figure 2b, recorded 10 min after exposure to the test solution, shows a sudden appearance of unusually large spontaneous potentials, the largest reaching 13.6 mV at an increased frequency of 4.40 s^{-1}. In three cells tested, average amplitudes (± s.d.) were increased from 0.6±0.1, 1.3±0.6 and 1.2±0.5 mV in normal solution to 7.1±4.3, 7.6±4.8 and 3.3±1.0 mV after exposure, respectively. We cannot readily explain, however, the appearance of the large MEPSPs. An anticholinesterase-like effect of CH_3HgCl can be ruled out, because the time course of the large MEPSPs was not prolonged. One possibility is that the compound may depolarise the preganglionic nerve terminals and trigger action potentials leading to release of large amounts of transmitter. At 15 min after exposure (Fig. 2c), the frequency of MEPSPs was further increased (in the three cells tested, 0.13, 0.13 and 0.12 s^{-1}, in normal solution, to 18.89, 21.20 and 25.60 s^{-1} after exposure, respectively). Some action potentials were initiated from the MEPSPs (Fig. 2c). Amplitude and configuration of the action potentials were similar to those evoked in normal solution by preganglionic nerve stimulation, indicating little effect of the compound on the postsynaptic cell membrane. At 20 min after exposure (Fig. 2d), the frequency gradually decreased.

Fig. 2 a–e, Application of CH_3HgCl, 0.04 mM, to a ganglion cell by perfusion. a, Normal solution; b–e, 10 min, 15 min, 20 min and 40 min after exposure. f–j $HgCl_2$, 0.04 mM. f, Normal solution; g–j, 30 min, 45 min, 80 min and 90 min after exposure. All records, except h and i, continuous from top to bottom. h and i, upper straight line shows zero potential. Calibration, 5 mV and 0.1 s for a, b. e, f, g and j; 10 mV and 0.1 s for c, d, h and i. See text for explanation. Records retouched.

At 40 min after exposure (Fig. 2e). MEPSPs ceased. At that time, the preganglionic nerve stimulation could not evoke any response in the ganglion cell, indicating that progress of the impulse to nerve terminals was blocked, and/or the transmitter substance was completely exhausted by the compound.

For comparison, similar experiments were carried out with an inorganic mercury compound $HgCl_2$ (0.04 mM; 1.34×10^{-5} g ml^{-1}) (Fig. 2f–j). $HgCl_2$ also caused increases in amplitude and frequency of the MEPSPs (Fig. 2g). Compared with CH_3HgCl, however, the increases were smaller and appeared after much longer exposure (more than 25 min). In three cells tested, amplitudes were increased from 1.5 ± 0.5 to 2.6 ± 1.2, 1.5 ± 0.9 to 3.4 ± 2.3 and 1.7 ± 0.4 to 2.0 ± 0.8 mV, and the frequencies from 0.28 to 6.14, 0.07 to 2.38 and 0.36 to 0.78 s^{-1} after exposure. At 45 min after exposure (Fig. 2h), the cell was gradually depolarised and underwent repetitive firing for more than 10 min. Further exposure (Fig. 2i) resulted in sustained depolarisation of the membrane to about 30 mV, and the firing ceased leaving only a local fluctuation (similar to a response) in the membrane potential. Eventually, 90 min after exposure, the cell became electrically silent (Fig. 2j).

Thus, it has been shown in in vitro experiments that the primary site of CH_3HgCl action is on the presynaptic nerve terminal, from which rapid and large spontaneous transmitter release occurs. $HgCl_2$ actions are both presynaptic, resulting in the release of relatively small quantities of transmitter, and postsynaptic, resulting in the sustained depolarisation of ganglion cells. In conclusion, mercury compounds, particularly CH_3HgCl, resemble the La^{3+} ion which is reported to cause also a rapid and large increase in the frequency of miniature endplate potentials in frogs[6].

We thank Professor I. Tanaka for guidance, Professors T. Morioka and T. Isa for advice, and Dr T. Inoue for CH_3HgCl.

M. S. JUANG
K. YONEMURA

Department of Physiology,
Kumamoto University Medical School,
Kumamoto, Japan

1 Takeuchi, T , in Minamata Disease, (edit. by Kutsuna, M.), 141 (Kumamoto University, Japan, 1968).
2 Bakir, F., et al., Science, 181, 230 (1973)
3 Miyakawa, T., and Deshimaru, M., Acta neuropath , 14, 126 (1969).
4 Miyakawa, T., et al , ibid , 15, 45 (1970).
5 Chang, L., and Hartman, H., Expl Neurol., 35, 122 (1972)
6 Heuser, J., and Miledi, R., Proc. R Soc Lond , B179, 247 (1971).
7 Weakly, J. N., J. Physiol., Lond , 234, 597 (1973).
8 Kajimoto, N , and Kirperker, S. M., Nature new Biol., 235, 29 (1972).
9 Balnave, R. J., and Gage, P. W., Br. J. Pharmac , 47, 339 (1973).
10 Blackman, J. G., Crowcroft, P J., Devine, C. E , Holman, M. E., and Yonemura, K., J Physiol., Lond., 201, 723 (1969).
11 Kanbayashi, K , Shirakawa, K , Hirota, K., Tsubaki, Y., and Tanaka, H , Igakuno ayumi, 91, 341 (1974) (in Japanese).
12 Von Burg, R , and Rustam, H , Electroencephalog. clin. Neurophysiol., 37, 381 (1974).

Chemotactic stimulation by cell surface immune reactions

CHEMOTAXIS of migratory leukocytes is of special interest to immunologists as it is intimately associated with tissue reactions initiated by immune responses. Chemotactic activity as measured in vitro with the Boyden chamber has been exclusively associated with chemotactic factors of various origins which all have in common the inherent ability to attract the responding cells. Immune-related chemotactic factors attracting neutrophils (polymorphonuclear leukocytes, PMNs) and macrophages are either complement (C)-derived as a result of C activation or they are products of activated lymphocytes. Thus, their activity is secondary to a specific humoral or cellular immune reaction taking place independent of and at a distance from the responding cell. Here we present a new concept of chemoattraction in which the chemotactic stimulus is a direct result of an immune reaction that proceeds on the surface of the responding cell itself. This concept originated from the discovery of the chemotactic stimulation of PMNs by cytotoxic sera at subcytotoxic doses.

This phenomenon was first observed in heterologous systems, nurse shark and dog sera cytotoxic for human PMNs (refs 1 and 2), and then for homologous systems, human and dog anti-sera against histocompatibility antigens that were cytotoxic for human and dog PMNs, respectively, (J A. J., and V. E., unpublished). The conditions in which chemotaxis was observed in these cytotoxic systems are shown in Fig. 1a, b and c. We interpreted these results to mean that cytotoxic immune reactions can cause an 'irritation' at the cell surface acting as a chemotactic stimulus provided at least one of the necessary reactants (antibody or the C system or even one limiting C component) is presented to the responding cell on a positive concentration gradient. In the following we show that cells carrying
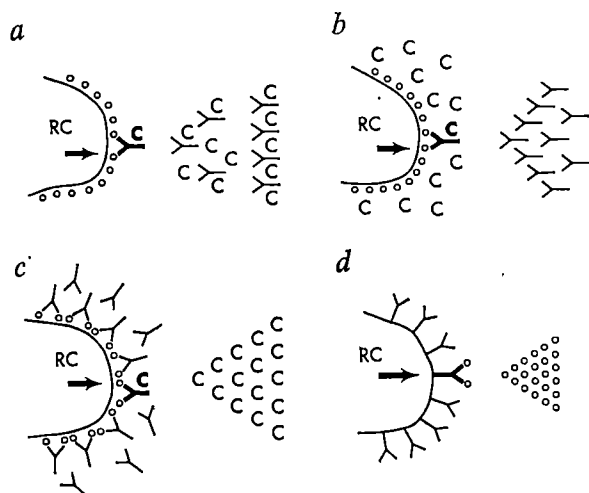
Fig. 1 Schematic representation of different modes of chemotactic stimulation by cell surface immune reactions. RC, Chemotactically responding cell (PMN, macrophage or lymphocyte); O, antigen; ⊀, antibody or recognition molecule; C, complement or limiting complement component; arrow, direction of chemotactic migration; symbols arranged as wedges, corresponding concentration gradient; symbols on cell surface, stimulating complex. *a–c*, Cytotoxic situations; *d*, antibody is cytophilic or, hypothetically, recognition antibody and the chemotactic gradient is formed by the antigen.

cytophilic antibody can be chemoattracted by the specific antigen. This situation could be of great biological significance, and is illustrated in Fig. 1*d*.

English short hair female guinea pigs (600 g) were immunised intracutaneously at three sites with a total of 3 mg crystalline ovalbumin in Freund's complete adjuvant. Two to three weeks later they were injected intraperitoneally with 30 ml 0.1% shell-fish glycogen in saline. After 4 h the animals were bled, killed and their peritoneal PMNs collected in heparinised saline. The cells were washed and, without further preparation, used in the chemotactic assay.

The cell preparations were usually 98% pure. The upper chamber compartment received 2×10^6 PMNs in Gey's medium.

From Table 1 the following conclusions may be drawn: PMNs from immunised animals were chemotactically attracted by the antigen, whereas those from controls were not. PMNs from normal, non-immunised animals, if treated with immune serum, behaved like cells obtained from immunised animals; if treated with preimmune serum they behaved like normal cells. Antigen introduced into the upper compartment of the chamber together with the cells did not increase random movement that could be mistaken for chemotaxis (R. Snyderman, personal communication). The chemotactic response was dependent on the antigen concentration (dose–response). Chemoattraction by the antigen could only be clearly demonstrated if normal guinea pig serum was present at low concentrations in the upper compartment (or in both compartments) of the chemotaxis chamber. Adding the serum to the upper compartment only had two major advantages: if the serum contained any chemotactic factors they would form a negative concentration gradient for the cells; and possible interactions of serum constituents with the chemotactic antigen were minimised.

We are not yet sure whether the requirement for serum in the medium represents C participation in the given circumstances. This possibility is under investigation. As regards the nature of the responsible antibodies, we have preliminary evidence that the responding cells, obtained from immunised animals or from normal animals and treated with immune serum, carry easily detectable antibody on their surface, whereas controls do not. We are probably dealing with cytophilic (homocytotropic) antibodies. It should be mentioned that the chemotactic response was antigen-specific, that is, that an unrelated antigen (bovine serum albumin, BSA) did not chemoattract the cells carrying anti-ovalbumin antibodies.

In considering the mechanism of this kind of chemotaxis, it is tempting to compare it with the phenomenon of hista-

Table 1 Chemotactic stimulation of guinea pig peritoneal immune PMN by the immunising antigen, ovalbumin

PMN (source, treatment)	CHP at antigen concentrations (μg/1.7 ml)							No antigen
	20	40	60	100	200	400	600	
IGP No. 17	3	7	34	45	102	102	68	18
IGP No. 22	72	209	184	457	267	164	—	79
IGP No. 22*	—	—	—	0	0	0	0	0
NGP No. 28	—	—	—	0	0	23	18	53
NGP No. 30	0	0	0	0	0	0	0	0
NGP No. 30, treated with No. 17 serum (1:10)	0	0	0	0	0	0	12	0
NGP No. 31	—	—	—	0	0	23	18	86
NGP No. 31, treated with No. 17 serum (1:5)	—	—	—	77	112	197	105	79
NGP No. 32	—	—	—	8	4	18	33	58
NGP No. 32, treated with No. 17 serum (1:5)	—	—	—	56	102	269	83	39
	Distance travelled (μm) at antigen concentrations (μg/1.7 ml)							
IGP No. 27	—	62	82	93	97	—	103	61
IGP No. 27, with antigen in upper compartment only	—	—	—	76	—	66	70	61
NGP No. 33	—	—	—	58	57	45	—	52
NGP No. 33, treated with pooled immune serum (1:5)	—	—	—	71	69	70	68	52
NGP No. 33, with antigen in upper compartment only	—	—	—	56	56	57	—	62

IGP, immunised guinea pig; NGP, normal (non-immunised) guinea pig; CHP, cells per high power field. *In vitro* chemotaxis was carried out and quantified as described[3]. Modified Boyden chambers were set up in triplicate. Results are expressed as average number PMN per high power field on lower surface of Millipore filters (5 μm pore size) separating the upper from the lower compartments of the chambers. Gey's medium was used throughout. Incubation time 3 h, temperature 37 °C in water saturated 5% CO_2 in air. Cells from non-immunised animals were divided and one half was treated for 45 min at 4 °C with homologous immune serum at the indicated final dilution in Gey's medium; the other half was treated in the same way with the corresponding preimmune serum. All cells were thoroughly washed in Gey's medium before they were placed in the upper compartments. Negative controls contained no antigen in the lower compartments. Chemotactic responsiveness of the cells was ascertained for each experiment. In all experiments except that marked by * the upper compartments contained 0.5% normal autologous guinea pig serum. The last five experiments (lower part of table) were quantified by measuring the distance (in μm) that the migrating cells had travelled into the filter[4].

mine release or degranulation exhibited by mast cells on contact of antigen with their cytophilic antibodies. The same kind of signal that induces this dramatic effect could well cause a migratory cell to move in the direction of its stimulated surface area.

We believe that this new concept may have several important implications under *in vivo* conditions: if wandering cells, carrying cytophilic antibody, can be attracted to the origin of antigen release, whether the antigen is microbial or derived from allogenic tissue or tumour cells, their accumulation at that site would then not only depend on C activation or the formation and release of chemotactic factors by proliferating lymphocytes. This mechanism, for instance, could contribute to the development of an Arthus reaction in C4-deficient guinea pigs (refs 5 and 6); it could also be the basis for the immune-mediated emigration of neutrophils into the lumen of the small intestine, observed in pigs sensitised with BSA (ref. 7)

We had an opportunity to test our views in clinical rather than experimental conditions A patient with a long history of dermatophytosis (*Trichophyton rubrum*) was by mistake skin-tested with an excessive dose of dermatophytin, and within 30 min developed a severe local reaction with increasing induration, erythema and pain accompanied by general malaise and fever. The reaction, which began to subside after 48 h, was Arthus-like, although there was no histological confirmation. The patient's peripheral PMNs isolated 2 and 8 weeks after the incident were strongly chemoattracted by dermatophytin, whereas PMNs from individuals negative to the skin test were completely unaffected by the antigen.

As preliminary experiments in our laboratory and elsewhere (P. C. Wilkinson, personal communication) conducted with macrophages are in agreement with the data reported here for PMNs, we believe that our concept of antigen chemotaxis may be a rather general one. We propose, therefore, as a hypothetical extension of our findings, that lymphocytes (of the B as well as the T-cell series) may also be chemoattracted to the site of the antigen that they are specifically equipped to recognise. The same signal that results in the turn-on of the cell on interaction of its surface recognition molecules with the antigen may also result in its directional migration. This hypothesis would endow the concept of immunological surveillance with an active search and guiding system instead of the chance encounter on which it is now based

This work was supported by the NIH

JOERG A. JENSEN
VIOLET ESQUENAZI

Department of Microbiology,
University of Miami, School of Medicine,
Miami, Florida 33152

1 Jensen, J. A , and Williams, D., *Nouv Rev fr D'Hémat.* 13, 889–891, 1973
2 Williams, D , thesis, Univ. Miami (1974).
3 Snyderman, R , Shin, H. S , Phillips, J. K., Gewurz, H., and Mergenhagen, S. E., *J. Immun.*, 103, 413–422 (1969)
4 Zigmond, S. H , and Hirsch, J G , *J. exp Med.*, 137, 387–410 (1973).
5 Jensen, J A , Garces, M C , and Iglesias, E , *Inf Immun* , 4, 12–19 (1971).
6 Frank, M M , Ellman, L., Green, I., and Cochrane, C. G , *J. Immun.*, 110, 1447–1451 (1973).
7 Bellamy, J E. C , and Nielsen, N. O., *Inf Immun* , 9, 615–619 (1974)

Control of acetylcholinesterase by contractile activity of cultured muscle cells

THE activity of the enzyme acetylcholinesterase (AChE) is one of the many properties of skeletal muscle known to be regulated by nerves. For example, AChE is synthesised and released by chick embryo muscle fibres grown *in situ* and in culture[1,2]. During maturation *in situ*, neural activity decreases AChE everywhere in the muscle fibres of chicks

except at the neuromuscular junction[3]. How nerves repress this extrajunctional AChE is not known. The possibility that they do so by signalling the muscles to contract, and that muscle contractile activity itself reduces the level of enzyme, has been tested by extracellular stimulation of muscle cultures with electrical impulses. Our results indicate that contraction reversibly reduces AChE activity of muscle cultures.

Muscle cultures were obtained from 11-day chick embryo pectoral tissue by trypsinisation and the cells were cultured in collagen-coated dishes in a medium of 10% horse serum, 2% embryo extract and 88% minimal essential medium (MEM)[2]. The fibres exhibited spontaneous contractions and cross striations after 5 d in culture and were readily maintained for 3 weeks *in vitro*.

Electrical stimulation resulted in decreases in the AChE of the cells and the AChE released into the medium (Table 1) In each of eight experiments the AChE activity was lower for stimulated than for unstimulated cultures. The average reduction of AChE activity of the cells was 37%. The decrease observed for the AChE released by the cells was even more pronounced, averaging 61%. The total decrease in AChE of the cultures varied with a range of 30% to 90%.

Since denervation increases AChE activity in chicken muscle fibres *in situ*[4], we investigated whether AChE activity would increase when electrical stimulation ceased. The data (Table 2) show that AChE increased after stimulation, indicating that suppression of AChE activity

Table 1 AChE levels of muscle cell cultures 48 h of stimulation

Experiment		ΔA per min per dish*	
		Cells†	Media‡
1	Unstimulated	0.62±0.05	3.21±0.40
	Stimulated	0.44±0.02	1.31±0.40
2	Unstimulated	0.36±0.02	2.41±0.20
	Stimulated	0.20±0.02	0.99±0.34
3	Unstimulated	0.36±0.06	1.29±0.20
	Stimulated	0.22±0.04	0.68±0.40
4	Unstimulated	0.17±0.03	0.89±0.13
	Stimulated	0.14±0.01	0.60±0.14
5	Unstimulated	0.19±0.02	1.27±0.20
	Stimulated	0.12±0.01	0.19±0.10
6	Unstimulated	0.33±0.02	1.51±0.25
	Stimulated	0.16±0.01	0.00±0.00
7	Unstimulated	0.38±0.02	1.20±0.20
	Stimulated	0.12±0.01	0.00±0.00
8	Unstimulated	0.36±0.05	1.71±0.50
	Stimulated	0.27±0.02	1.33±0.15
Average	Unstimulated§	0.35	1.69
	Stimulated	0.21	0.64

A Grass S8 stimulator was connected in series to a pair of platinum electrodes approximately 25 mm long and 3 mm wide. The electrodes were attached to the top of a culture dish and immersed in the culture media. Six dishes in series were given a 100 mm s⁻¹ train consisting of three 10-ms pulses repeated every 5 s. The voltage was approximately 30 V, the minimum that produced contractions of >75% of the fibres as determined with an inverted phase contrast microscope. Stimulation was continued for 48 h and polarity was switched frequently to minimise effects of electrolysis.
*AChE activity was determined spectrophotometrically by the Ellman assay[10] using the pseudocholinesterase inhibitor iso-OMPA as in previous reports [1–3].
†Values are averages ± s.d. of six dishes for experiments 1 and three dishes for experiments 2–8.
‡Values are averages ± s.d. of 12 dishes for experiment 1 and six dishes for experiments 2–8.
§Statistically significant difference between unstimulated and stimulated cultures $P < 0.01$ (Student's *t* test for paired variates[11]).

Table 2 AChE levels of muscle cell cultures 48 h after stimulation

Experiment		ΔCell†	ΔA per min per dish* media	Total production
1	Unstimulated	+0.21±0.14	3.09±0.30	3.30±0.44
	Stimulated	+0.10±0.08	1.24±0.04	1.34±0.12
2	Unstimulated	−0.14±0.03	1.02±0 20	0.88±0.23
	Stimulated	+0.15±0.05	1.23±0.30	1.38±0.35
3	Unstimulated	−0.18±0.08	0.56±0.07	0.38±0.15
	Stimulated	+0.22±0.08	1.62±0.20	1.84±0.28
4	Unstimulated	0.00±0.04	0.62±0.04	0.62±0.08
	Stimulated	+0.08±0.02	0.99±0.10	1.07±0.12
5	Unstimulated	0.00±0.04	1.33±0.27	1.33±0.31
	Stimulated	+0.10±0.03	1.32±0.06	1.42±0.09
7	Unstimulated	−0.20±0.09	1.08±0.10	0.88±0.19
	Stimulated	+0.25±0.05	2.23±0.07	2.47±0.12
8	Unstimulated	−0.10±0.09	1.09±0.20	0.99±0.29
	Stimulated	+0.01±0.04	1.30±0.05	1.31±0.09
Average				
	Unstimulated‡	−0.06	1.26	1.20
	Stimulated	+0.13	1.42	1.55

*Values are means of ± s.d. of six dishes for experiment 1 and three dishes for experiments 2–8.

†Cell activity 48 h after stimulation minus cell activity immediately after stimulation.

‡Statistically significant difference between unstimulated and stimulated cultures $P<0.02$ for experiments 2–8. Differences are not significant ($P>0.10$) for experiments 1–8. (Student's t test[11].)

was reversible. In addition, except for the first experiment, cell AChE and release of AChE in the medium tended to be greater 48 h after stimulation for previously stimulated than for unstimulated cultures.

The increase of AChE activity after stimulation was also evidence that stimulation did not injure the cells. Furthermore, the cells were still contracting and were morphologically unchanged when observed by light microscopy after 48 h of stimulation. Also, the ability of the cultures to synthesise protein was not affected. Incorporation of ^{3}H-leucine[2] (ICN Pharmaceuticals, 10 μCi per culture in leucine-free MEM) into trichloroacetic acid precipitates of cultures labelled for 1 h after stimulation gave an average in four experiments of 38,000 c.p.m. for stimulated cultures and 36,000 c.p.m. for unstimulated cultures.

We reasoned that if muscle activity were involved in regulating AChE, cultures unable to contract would produce more AChE than those contracting spontaneously Tetrodotoxin (a drug that blocks excitation of the membrane of muscle fibres in culture[5]), 0.1 mg per dish, was added to 5-d-old cultures and the cells were maintained in a non-contracting state for 6 or 8 d. The cells and media were sampled every 2 d and the total AChE produced over the entire period was compared for the relaxed and the contracting cultures (Table 3). The non-contracting tetrodotoxin-treated cultures produced much larger amounts of AChE, averaging 218% the activity of the spontaneously contracting cultures.

Stimulation has also been shown to affect ACh sensitivity *in situ* and ACh receptors in culture. Drachman and Witzke[6] and Lømø and Rosenthal[7] reduced the spread of ACh sensitivity over the membrane of denervated rat muscles and Cohen and Fischbach[5] reduced the extrajunctional ACh receptors of embryo muscle cultures by means of electrical stimulation. When considered together, these reports and the data presented here strongly suggest that some regulatory actions of nerve on muscle are mimicked by stimulation and may be due to muscle activity. particularly with regard to extrajunctional localisation of

Table 3 Effect of tetrodotoxin on AChE of muscle cell cultures

Experiment		ΔA per min per dish accumulated activity*	Interval (d)
9	Untreated	10.5±1.1	8
	TTX	29.9±2.5	
10	Untreated	14 9±1.6	8
	TTX	25.4±1.0	
11	Untreated	10.4±1.1	6
	TTX	20.4±1.7	
Average % TTX per untreated		218	

Tetrodotoxin (TTX, 0.1 µg per dish) was added to cultures at 5 d and maintained for the time indicated.

*Values for averages ± s.d. of five dishes for experiment 9 and four dishes for each experiments 10 and 11. Accumulated activity represents the sum of the changes in AChE activity of the cells plus the activity released into the medium during 6 or 8 d.

molecules involved in transmission at the neuromuscular junction. Whether contractions themselves are necessary or whether excitation of the muscle membrane would suffice is a matter for further investigation. There is evidence that muscle activity[8] and cholinergic transmission[9] may not be required for localisation of ACh receptors at the neuromuscular junction. Therefore, it is possible that there are separate regulatory mechanisms for extrajunctional and endplate AChE and ACh receptors.

C. R. WALKER
B. W. WILSON*

*Department of Avian Sciences,
University of California at Davis,
Davis, California 95616*

1 Wilson, B. W, Linkhart, T. A, Walker, C. R, and Nieberg, P. S., *J. Neurol. Sci*, 18, 333–350 (1973)
2 Wilson, B. W., Nieberg, P. S, Walker, C R, Linkhart, T. A., and Fry, D M., *Devl Biol*, 33, 285–299 (1973)
3 Wilson, B. W., and Walker, C. R., *Exploratory Concepts in Muscular Dystrophy II* (edit by Milhorat, M D) (Excerpta Medica, in the press).
4 Wilson, B. W, Kaplan, M. A., Merhoff, W. C, and Mori, S. S., *J. exp. Zool.*, 174, 39–54 (1970).
5 Cohen, S. A., and Fischbach, G. D., *Science*, 181, 76–78 (1973).
6 Drachman, D. B, and Witzke, F, *Science*, 187, 514–516 (1972).
7 Lømø, T., and Rosenthal, J., *J Physiol*, 221, 493–513 (1972).
8 Steinbach, J H, *Nature*, 248, 70–71 (1974).
9 Steinbach, J. H, Harris, A. J, Patrick, J, Schubert, D, and Heinemann, S O, *J gen Physiol.*, 62, 255–270 (1973).
10 Ellman, G. L., Courtney, D. D., Andres, V., and Featherstone, R. M., *Biochem. Pharmacol*, 1, 88–95 (1961).
11 Alder, H L, and Roessler, E B, *Introduction to Probability and Statistics.* (Freeman, San Francisco, 1968)

Presence in brain of a mitogenic agent promoting proliferation of myoblasts in low density culture

NORMAL animal cells in tissue culture proliferate very slowly, or not at all, when their density is less than 20 cells mm^{-2} (ref. 1). This property of density-dependent cell proliferation has long been a major obstacle to establishing clonal cultures. Conditioned media[4] or feeder layers[2,3] have been used to promote proliferation of single cells in culture. We have reported the presence, in bovine pituitary and brain, of a mitogenic factor, fibroblast growth factor (FGF), that can control the rate of growth of animal cells *in vitro*[5] We report here that the proliferation of myoblasts
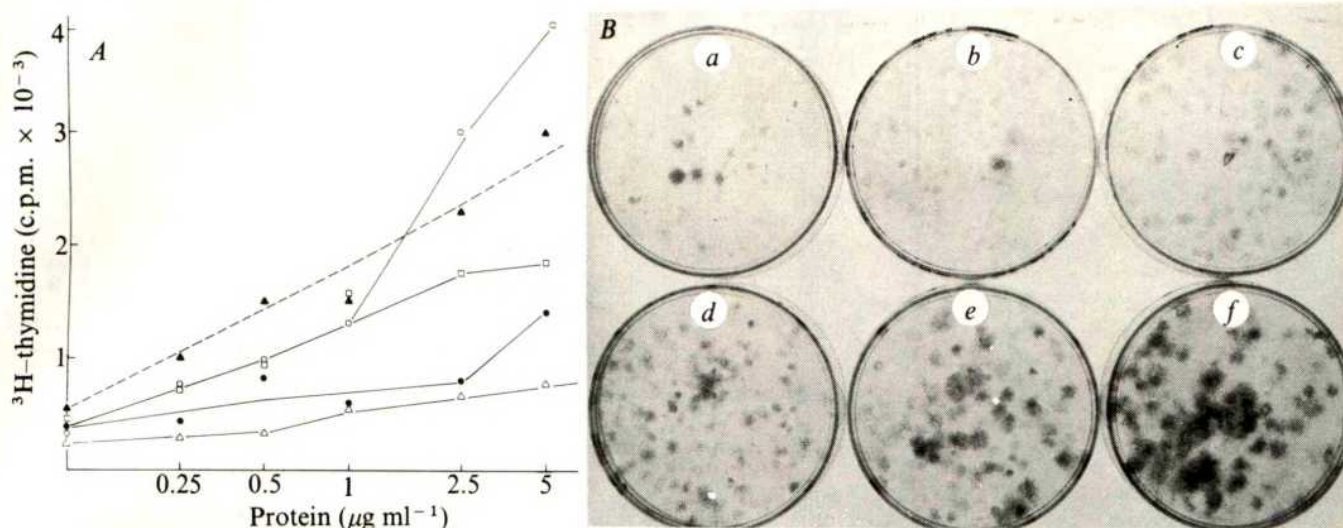
Fig. 1 *A*, DNA synthesis in BALB/c 3T3 cells in response to various concentrations of brain crude extracts prepared at diverse *p*H. Crude brain extracts were produced by homogenisation of bovine brains at 4 °C in 0.15 M $(NH_4)_2SO_4$. The *p*H was adjusted to either 3.0, 4.0, 4.5, 7.0 or 8.5 and the homogenate was stirred for 2 h at 4 °C, and centrifuged at 23,000*g*. The supernatant was dialysed, centrifuged and then lyophilised. FGF was prepared as described previously[5,6]. 3T3 cells were plated in Dulbecco's modified Eagle's medium (DME) with 10% calf serum (4 × 10⁴ cells per 35 × 15 mm dish with 2 ml medium per dish). After 1 d the medium was removed and replaced by DME with 0.1% calf serum. Two days later various concentrations of samples dissolved in the presence of 0.5% crystalline bovine serum albumin (to minimise nonspecific adsorption) were added to the cultures. Determinations of the incorporation of ³H-thymidine into DNA were carried out as described previously[5]. ○, *p*H 4.0; ▲, 4.5; □, 3.5; ●, 7.0; △, 8.5. *B*, Effect of brain crude extracts obtained at different *p*H on the proliferation of myoblasts seeded at low density. Myoblasts were obtained from foetal calf (at 3 months gestation) by trypsinisation of the thigh muscle as described previously[12]. The resulting cell suspension was plated on to three 15-cm plastic tissue culture dishes (Falcon) in 1:4 DME–medium 199 supplemented with 10% foetal calf serum and 1% chick embryo extract, then incubated at 37 °C in a humidified incubator in an atmosphere of 5% CO_2 in air. After 1 d the medium was renewed and 5 µg ml⁻¹ cytochalasin B was added to select for myoblasts as described by Sanger[13]. The next day, the myoblasts were shaken loose from the dishes by gentle agitation, and the resulting suspension was centrifuged. The pellet was suspended in 1:4 DME–medium 199 with 10% horse serum. The cells were then plated at a final concentration of 300 cells per 6-cm gelatinised dish in 5 ml 1:4 DME–medium 199, with 10% horse serum. The brain crude extracts obtained at diverse *p*H were added 24 h later to a final concentration of 100 µg ml⁻¹. Similar numbers of clones were observed when the extract was added at the time of seeding or 1 d later. The dishes were incubated for 14 d (control) and 7 d (experimental) without a change of medium or further additions. After rinsing and fixing, the dishes were stained with crystal violet. *a*, Control; *b*, *p*H 3.5; *c*, 4.0; *d*, 4.5; *e*, 7.0; *f*, 8.5. Note the formation of myotubes in *e* and *f*.

seeded at low densities can be greatly accelerated by low concentrations of brain or pituitary crude extracts. This myoblast growth factor (MGF) is distict from FGF.

The effect of brain crude extracts prepared at diverse *p*H on the initiation of DNA synthesis in 3T3 cells maintained in low serum is shown in Fig. 1*a*. Extracts prepared at *p*H 4.0 or 4.5 were the most potent, and those prepared at *p*H 7.0 and 8.5 were essentially free of activity. In contrast, when the diverse crude extracts were tested on sparse, primary cultures of myoblasts maintained in 10% horse serum, the most active extract was that prepared at *p*H 8.5. The *p*H 7.0 extract was less active than the *p*H 8.5 (Fig. 1*b*).

As the mitogenic (FGF) activity for 3T3 cells is extracted preferentially at *p*H 4.5, whereas the activity responsible for the growth of myoblasts maintained at very low density in the presence of 10% serum is best extracted at *p*H 8.5, the activities are, very likely, distinct.

Ion-exchange chromatography provided further evidence that MGF activity was distinct from FGF. When a *p*H 8.5 crude extract of brain was applied on a CM Sephadex column equilibrated with 0.1 M sodium phosphate, *p*H 6.0, MGF activity was unadsorbed. In the same conditions, the FGF activity was retained[6]. When the *p*H 8.5 crude extract of brain was applied on a DEAE-Sephadex column equilibrated with 0.05 M sodium phosphate, *p*H 7.6, MGF activity was retained and the FGF activity was unadsorbed. This indicates that MGF is a neutral polypeptide compared with FGF, which is basic.

The effect of the *p*H 8.5 brain crude extract on the rate of proliferation of myoblasts seeded at 300, 100 and 50 cells per 6-cm dish was measured (Fig. 2). After 14 d, little growth had taken place in the control, although the cells had been maintained in the presence of 10% horse serum. In contrast, when the myoblasts were maintained in 10%

horse serum plus 100 µg ml⁻¹ of *p*H 8.5 extract, extensive proliferation took place in all cases. Extensive differentiation, as reflected by the formation of myotubes, was also observed (Fig. 3). In 70% of the colonies, striated myotubes formed and in 20% of the colonies, syncytium formation was noticed. This suggests that crude extract not only promotes cell proliferation, but also promotes differentiation, as more than 75% of the nuclei were in myotubes.

When the myoblasts were maintained in the presence of FGF, proliferation was stimulated, but not to as great an extent as with crude extract of brain. Furthermore, the colonies did not differentiate into myoblasts as extensively as those with brain crude extract added. When myotubes were present in a colony they did not contain more than two to five nuclei per myotube (Fig. 3).

Pituitary crude extracts had an effect similar to that of brain crude extracts and FGF purified from pituitary had an effect similar to FGF from brain.

Chick embryo extract promoted the proliferation of myoblasts seeded at low density, although the effect was small when compared with brain crude extract (Fig. 4).

As NIH pituitary preparations, such as luteinising hormone (LH) and thyroid-stimulating hormone (TSH), have been reported to contain mitogenic contaminants[7,8], the effects of several NIH preparations on myoblast proliferation were compared. Of the various preparations tested, follicle-stimulating hormone (FSH) and gonadotrophic hormone had the least activity. LH of either ovine or bovine origin and TSH, however, had considerable growth-promoting activity. Insulin, which has been reported to promote cell proliferation *in vitro* when present at high concentrations, was inactive on myoblasts seeded at low density (Fig. 4).

In conclusion, we have reported that crude extract of
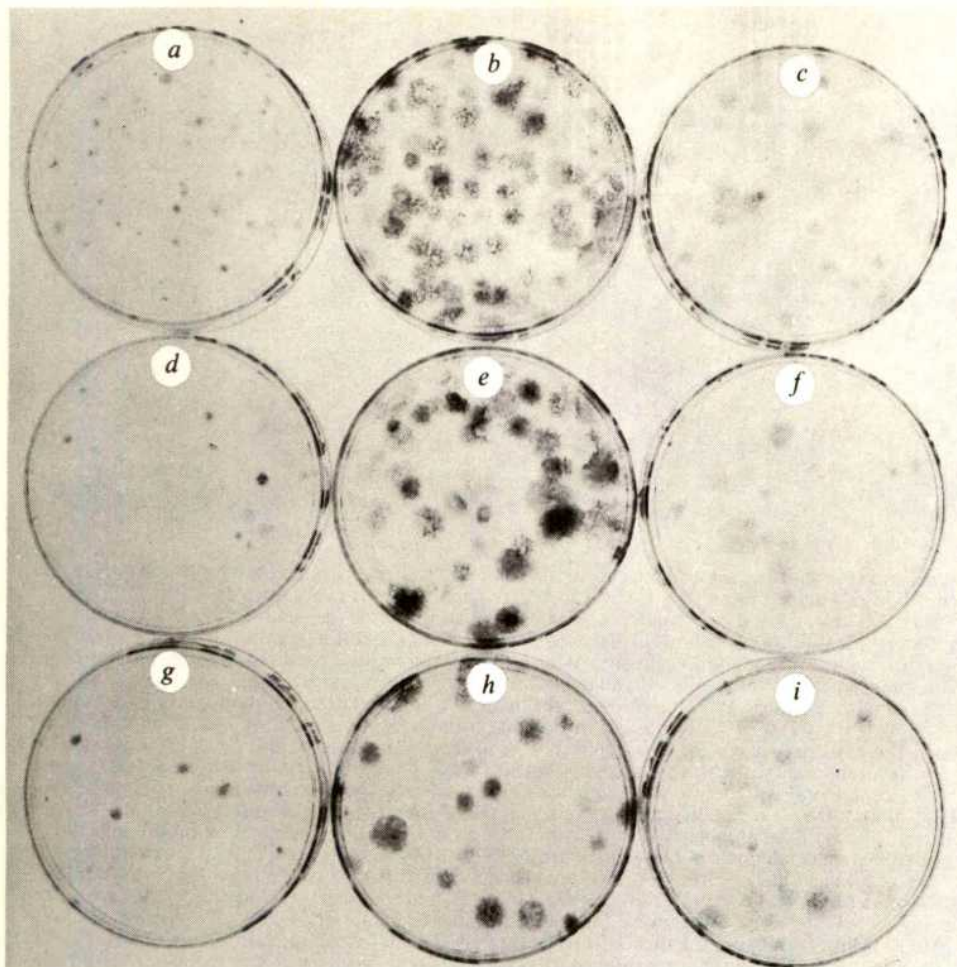
Fig. 2 Comparison of effects of brain crude extract (*p*H 8.5) and FGF on proliferation of myoblasts seeded at low density. Cells (300, 100 or 50) were plated in 6 cm dishes in 5 ml 1:4 DME–medium 199 with 10% horse serum. Brain crude extract (100 μg ml^{-1}) and FGF (1 μg ml^{-1}) were added 24 h later. Controls did not receive any additions and no change of medium was carried out during the incubation. Controls were incubated for 7 d. After 7 d, the myotubes in the dishes in the presence of brain crude extract started to contract rythmically and detached, preventing further incubation. *a*, 300 cells; *b*, 300 cells plus 100 μg ml^{-1} brain extract, *p*H 8.5; *c*, 300 cells plus 1 μg ml^{-1} FGF; *d*, 100 cells; *e*, 100 cells plus 100 μg ml^{-1} brain extract, *p*H 8.5; *f*, 100 cells plus 1 μg ml^{-1} FGF; *g*, 50 cells; *h*, 50 cells plus 100 μg ml^{-1} brain extract, *p*H 8.5; *i*, 50 cells plus 1 μg ml^{-1} FGF. Note the formation of myotubes in *b*, *e* and *h* and their apparent absence in *c*, *f* and *i*.

brain prepared at *p*H 8.5 contains potent mitogenic factor(s) for myoblasts seeded at low density. The observation that those factors for myoblasts can be selectively extracted at high *p*H, whereas FGF, soluble at low *p*H, is reminscent of the situation for polypeptide hormones; some are most soluble at low *p*H, whereas others are most soluble at high *p*H (ref.9).

In addition to its mitogenic effect, the *p*H 8.5 brain crude extract also promoted the formation of myotubes. In that respect it seems to act as a differentiating agent similar to those present in conditioned medium used for promoting

the fusion of chick myoblasts[10,11]. In contrast, FGF, although it was less potent than the *p*H 8.5 crude extract, promoted proliferation of myoblasts but not extensive fusion. This observation agrees with the suggestion that FGF is an agent which promotes proliferation but delays fusion (our unpublished work).

The observation that, at *p*H 8.5, additional mitogenic agents other than FGF can be extracted from the brain may be of technical use for cloning cells or for accelerating the growth of the cells that divide slowly.
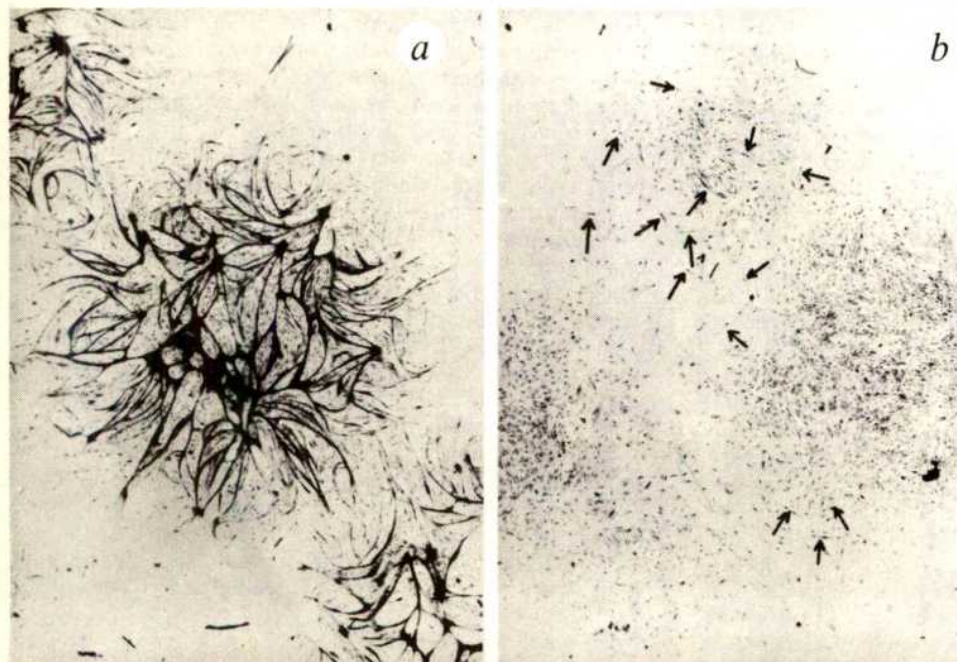
Fig. 3 Clones of myoblasts (×10) grown in presence of either brain crude extract, *p*H 8.5, or FGF. *a*, Clone grown in the presence of brain crude extract, pH 8.5 (100 μg ml^{-1}). Cells (300) were seeded in a 6 cm dish giving rise to colonies. Shown is a clone arising from one of these cells after 10 d. Note extensive network of myotubes, very well differentiated. Some of the myotubes contain as many as 1,000 nuclei. In some instances, presence of a loose syncytium was observed. *b*, Clone grown in the presence of FGF (1 μg ml^{-1}). Original plating was as described for *a*. Clone contained mostly single cells, with some forming short myotubes with two to three nuclei (arrows).

Fig. 4 Comparison of effect of chick embryo extract, insulin and NIH hormone preparations on the proliferation of myoblasts seeded at low density. Cells (300) were plated in 6 cm dishes in presence of 5 ml 1:4 DME–medium 199 with 10% horse serum. *a*, Control; *b*, 10% chick embryo extract; *c*, 1% chick embryo extract; *d*, 0.1% chick embryo extract; *e*, insulin (1 μg ml⁻¹); *f*, NIH FSH S9 (100 μg ml⁻¹); *g*, NIH ovine growth hormone (100 μg ml⁻¹); *h*, NIH LHB8 (100 μg ml⁻¹); *i*, NIH LH S17 (100 μg ml⁻¹); *j*, NIH TSH B6 (100 μg ml⁻¹). Plates were incubated for 14 d (control) and 7 d (experimental). After washing and fixing, the plates were stained with crystal violet.
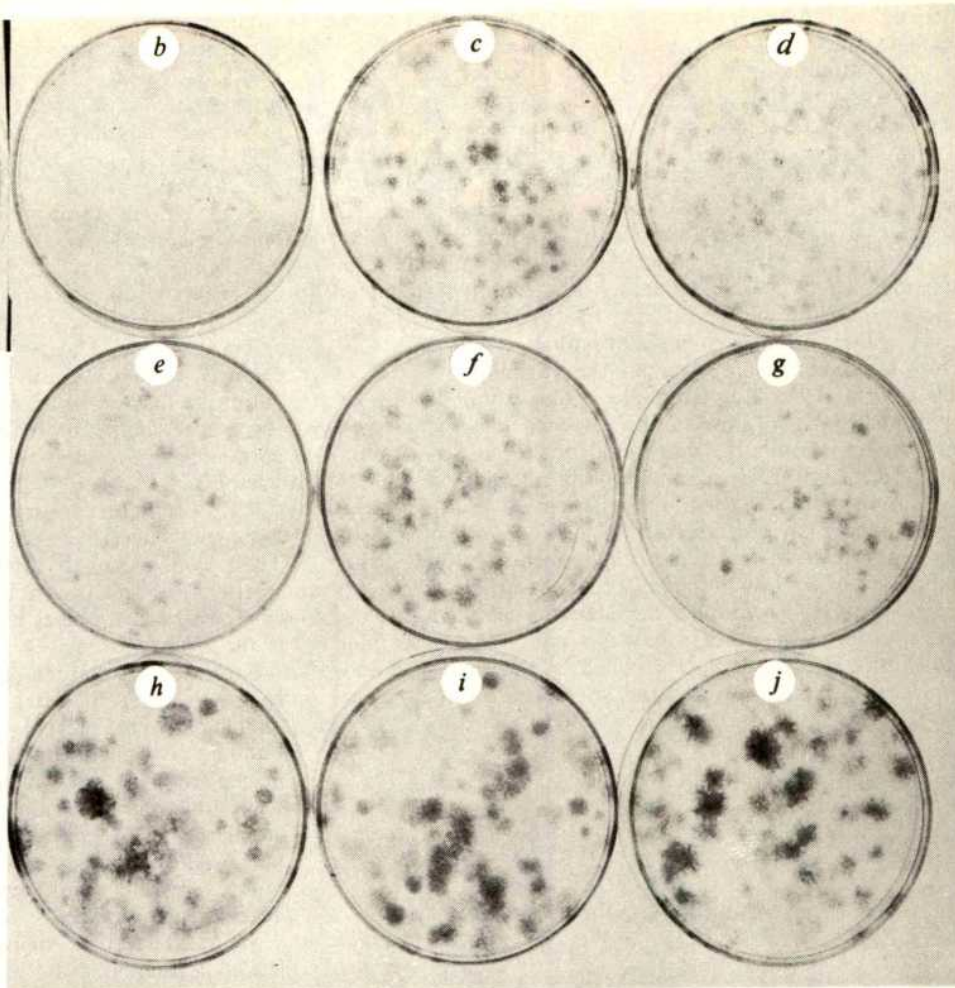
D. GOSPODAROWICZ
J. WESEMAN
J. MORAN

*The Salk Institute for Biological Studies,
PO Box 1809, San Diego, California 92112*

1 Harris, H., *Br. J. exp. Pathol.*, **36**, 115 (1955).
2 Sanford, K. K., Earle, W. R., and Likely, G. D., *J. natn. Cancer Inst.*, **9**, 229 (1948).
3 Rubin, H., *Expl Cell Res.*, **31**, 138 (1966).
4 Puck, T. T., Marcus, P. I., and Cieciura, S. J., *J. exp. Med.*, **103**, 273 (1956).
5 Gospodarowicz, D., *Nature*, **249**, 123 (1974).
6 Gospodarowicz, D., *J. biol. Chem.*, **250**, 2515 (1975).
7 Holley, R. W., and Kiernan, J. A., *Proc. natn. Acad. Sci. U.S.A.*, **60**, 300 (1968).
8 Corvol, M., Malemud, C. J., and Sokoloff, L., *Endocrinology*, **90**, 262 (1972).
9 Ellis, S., *Endocrinology*, **69**, 554 (1961).
10 Konigsberg, I. R., *Science*, **140**, 1273 (1963).
11 White, N. K., and Hauschka, S. D., *Expl Cell Res.*, **67**, 479 (1971).
12 Buckingham, M. E., Caput, D., Cohen, A., Whalen, R. G., and Gros, F., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1466 (1974).
13 Sanger, J. W., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3621 (1974).

Capacity of foetal spinal cord obtained from dystrophic mice (*dy²J*) to promote muscle regeneration

RECENTLY Gallup and Dubowitz[1] described a series of tissue culture experiments in which they attempted to clarify the part played by the motor neurone in the pathogenesis of murine muscular dystrophy. Bishop *et al.*[2] had shown that muscle explants obtained from human subjects grew nor-

mally in Carell flasks. Gallup *et al.*[3] reported similar results with muscle explants of trypsinised minced muscle obtained from dystrophic chick that had been grown *in vitro* on collagen-coated surfaces in Petri dishes. They were unable to differentiate between cultured normal and dystrophic muscle. But they reported significant differences between cultures of normal mouse cord with either normal or dystrophic muscle and dystrophic cord with either normal or dystrophic muscle. When contacted by ventral root neurites from normal spinal cord, the muscle cells of both normal and dystrophic muscle fused to form new fibres with well developed cross striations and peripherally located nuclei. The appearance of both normal and dystrophic muscle coupled with 'dystrophic' spinal cord was quite different. The initial cellular regenerative response was followed in most cases by degeneration and disappearance of all muscle elements, leaving only strands of connective tissue. In only three out of thirteen cultures were myotubes formed, and these failed to develop synchronised contractions.

On the basis of this evidence, Gallup and Dubowitz[1] concluded that the lesion causing murine dystrophy in muscle is primarily neurogenic.

Their results partly contradict our own observations[4] that normal and dystrophic muscle can regenerate equally well in the presence of foetal spinal cord obtained from either normal or dystrophic mice. In an attempt to explain the apparent contradiction, we have examined the procedure applied by Gallup and Dubowitz[1]. It occurred to us that Gallup and Dubowitz[1] may possibly be reporting results obtained in their small samples mainly from cultures of lumbar spinal cord. In our previous investigations we randomly coupled cross sections from all levels of the cord

with adult muscle. It seemed imperative, therefore, to determine whether the suspected neurogenic lesion of the dystrophic mutant might initially be located in the lumbo-sacral portion of the cord. Since the dystrophy in the *dy* mouse is progressive and starts in the muscles of the hind limbs which are innervated by lumbo-sacral fibres, the possibility that a difference in the procedures may be responsible for the contradictory results could not be excluded.

To test this hypothesis, cervical, thoracic and lumbar foetal cord from 15-d-old mouse embryos homozygous for dystrophy dy^2J/dy^2J were explanted and within 5 d coupled to normal tibialis muscle as well as to dystrophic tibialis muscle obtained from young adult mice.

Our attention was focused on the capacity of different levels of spinal cord to stimulate regeneration of muscle. In particular, the purpose of this experiment was to determine whether the lumbar and sacral portion of 'dystrophic' cord differs from cervical and thoracic cord in its ability to promote differentiation of regenerated muscle strips coupled with it in culture.

The experimental technique relied essentially on a tissue culture system in which spinal cord and muscle fragments from normal and dystrophic mice were explanted in close proximity to each other, that is, 'coupled'. Dystrophic mutant mice C57BL/6J dy^2J supplied by the Jackson Laboratory, Bar Harbor, Maine, and raised in our laboratory were used.
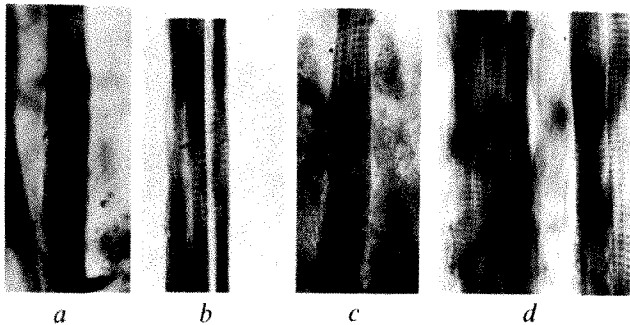
Fig. 1 *a*, Whole mount preparation of a culture of normal muscle coupled to dystrophic cord cervical level *in vitro* 50 d with phosphotungstic acid haematoxylin stained (PTAH); ×380. *b*, Same as *a* coupled to dystrophic cord thoracic level, 50 d *in vitro*, stained with PTAH; ×380. *c*, Same as *a* coupled to dystrophic cord lumbar level, 60 d *in vitro*, stained with PTAH; ×380. *d*, Same as *a* coupled to dystrophic cord sacral level, 60 d *in vitro* stained with PTAH; ×380.

Spinal cords were dissected from foetal mice aged 13–14 d *in utero*. After laminectomy, cervical and lumbar cord with its meningeal covering and attached dorsal root ganglia (DRG) were obtained from the foetuses. Complete cross sections of the spinal cord with their occompanying DRG (ref. 5) were explanted on to collagen-coated coverslips[5,6] provided with a drop of nutrient fluid, and sealed into a Maximow double coverslip assembly. The nutrient medium which was renewed twice a week consisted of 33% human placental serum, 10% chick embryo extract (9 d), 50% Eagle's minimal essential medium (MEM), 7% balanced salt solution, and was supplemented with 600 mg% glucose and 1.32 ml⁻¹ of acromycin. Cultures were incubated at 34–35 °C in the lying-drop position, and observed regularly by bright field microscopy employing a long-working distance, ×40, oil immersion objective.

Phenotypically dystrophic mice were obtained from matings between tested homozygotes for the dystrophic gene dy^2J. Young adult mice homozygous for dystrophy and normal mice served as donors for muscle explants. Strips of muscle fibres were explanted near 4- to 5-d-old spinal cord cultures[5,6]. The following combinations were

studied: (1) normal cords and normal muscle; (2) cervical, thoracic, lumbar and sacral levels of cord obtained from foetuses homozygous for the *dy* mutations coupled to normal tibialis muscle (Fig. 1).

Normal muscle coupled with either normal or *dy* foetal spinal cord regenerated in culture. Innervation of the muscle explants led to gradual increases in fibre size and the development of cross striations. Spontaneous synchronised contraction occurred intermittently.

Fragments from all levels of the spinal cords from dystrophic foetal mice were as effective as were normal cords in inducing regeneration and innervation of muscle.

These results clarify some of the conflicting evidence reported in the literature. They confirm our earlier observations[4], that both normal and dystrophic muscle can regenerate in the presence of either normal or dystrophic cord. These results were most recently confirmed by Parson[7].

The conflicting observations reported by Gallup and Dubowitz[1] may be due to a statistical sampling error. Their conclusion that muscular dystrophy is determined solely by the spinal cord and that the neurones in the spinal cord are by themselves capable of producing the myopathy is far from proven.

Several investigators have reported that electron microscopy *in vitro* material revealed abnormalities in motor endplates were present, however, both in muscle fibres numbers and distortions of synaptic vesicles[8]. In cultures of normal or dystrophic muscles coupled to *dy* cord we have seen similar motor endplate abnormalities[4]. Such abnormal endplates were present, however, both in muscle fibres which displayed extensive cross striations and highly ordered myofilaments, and in muscle fibres which showed degenerative changes.

We tentatively conclude, therefore, that the primary lesion in muscular dystrophy is probably myogenic rather than neurogenic. Our experiments do not, however, permit us to decide with certainty between these two alternatives. The tissue culture set up may not be as well suited as originally anticipated to analyse these problems since there are strong indications that in the tissue culture environment both the penetrance and the expressivity of the dystrophic alleles are considerably diminished.

Max Hamburgh
Edith Peterson
Murray B. Bornstein
Cassandra Kirk

*Saul R. Korey Department of Neurology,
Department of Anatomy,
Albert Einstein College of Medicine,
Yeshiva University,
Bronx, New York 10461
and Department of Biology,
City College of New York, New York*

1 Gallup, B., and Dubowitz, V., *Nature*, 243, 287–289 (1973).
2 Bishop, A., Gallup, B., Skeate, Y., and Dubowitz, V. *J. neurol. Sci.*, 13, 333 (1971).
3 Gallup, B., Strugalska-Cynowska, H., and Dubowitz, V., *J. neurol. Sci.*, 17, 109 (1972).
4 Hamburgh, M., *et al.*, *Progr. Brain Res.*, 46, 479–508 (1973).
5 Peterson, E. R., Crain, S. M., and Murray, M. R., *Z. Zellforsch.*, 66, 103–154 (1965).
6 Peterson, E. R., and Crain, S. M., *Expl Neurol.*, 36, 136–159 (1972).
7 Parson, R., *Nature*, 251, 621–622 (1934).
8 Pachter, B., Davidowitz, T., and Breinin, M. B., *Proc. 32nd Ann. Mtg Electron Micr. Soc. Am.*, 6 (1974).

Cross bridges as the major source of compliance in contracting skeletal muscle

It has been established that the elastic properties of contracting skeletal muscle are dominated by structures within the sarcomeres We have demonstrated[1] a decrease in the instantaneous stiffness of a contracting toad sartorius at lengths greater than body length (l_0) coinciding directly with the decline in maximum isometric tetanic tension. This finding suggested that the cross bridges between actin and myosin filaments may be the major site of compliance in an active muscle, a conclusion in accordance with that reached by Huxley and Simmons[2] on the basis of a study of tension transients following rapid releases in single fibres of frog semitendinosus. Also in agreement with this conclusion is the work of Cleworth and Edmond[3] who used a light diffraction technique to demonstrate that any change in the length of the sarcomere during isometric contraction was less than 50 Å, or less than 0.1%. These studies suggest that the cross links could be the major site of elasticity within the sarcomere, but it is possible that significant compliance resides in the unbonded thin filaments, although X-ray diffraction studies of contracting muscle[4] indicate that the I-filament length does not increase by more than 0 02%. The experiments reported here were carried out to extend previous observations of muscle stiffness into the range of lengths below the resting length.

All experiments to be reported here were carried out using whole sartorii of the toad *Bufo bufo* The method used for obtaining the tension–extension curve by constant velocity releases was as described previously[1], using a release velocity of 1.4–2.5 l_0 s^{-1} (V_{max} for toad muscle was measured and found to be 1.27 l_0 s^{-1})[1].

For comparison of results from different experiments, all force values were normalised with respect to maximum tetanic force (P_0), and the changes in length were normalised with respect to body length (l_0). Plotting force against change in length produces a tension–extension curve, the linearity of a portion of which enables a linear regression

Fig. 1 Tension–extension curves from maximum tetanic tension in toad sartorius at four different lengths: ●, l_0; ▲, $0.8l_0$; ○, $0.72l_0$; ■, $0.68l_0$. The stiffness values for these curves are $77P_0/l_0$ (l_0 curve), $63P_0/l_0$ ($0.8l_0$ curve), $50P_0/l_0$ ($0.72l_0$ curve), $37P_0/l_0$ ($0.68l_0$ curve). For this muscle $l_0=25$ mm; weight$=67$ mg.
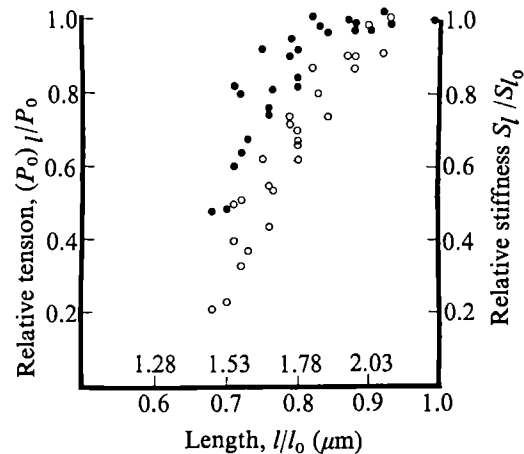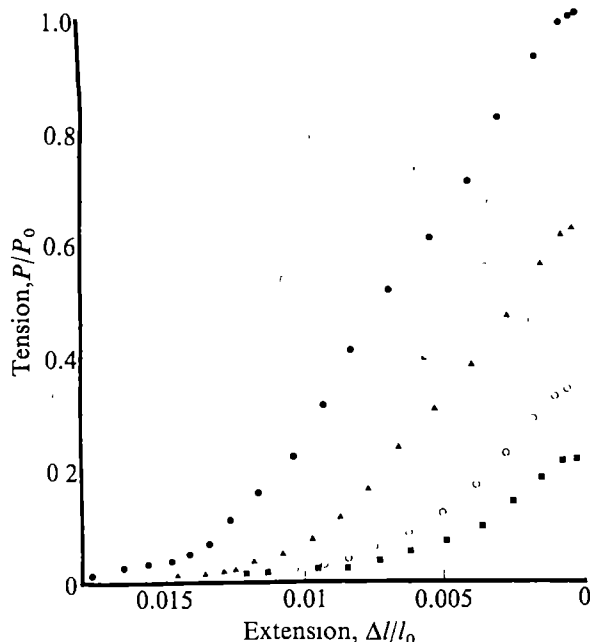
Fig. 2 Comparison of instantaneous stiffness and maximum tetanic force below body length in toad sartorii. Stiffness values (●) and tension values (○) represent the pooled data from six separate experiments. Stiffness and tension are normalised with respect to their maximum values at body length. Length is expressed as a fraction of l_0. Abscissa: sarcomere width at the corresponding length.

analysis of the points from $0.4P_0$ to P_0 and the use of the slope of the line as a measure of muscle stiffness[1].

Four tension–extension curves obtained from the same muscle at different lengths below l_0 are shown in Fig. 1. The stiffness of the contracting muscle is decreasing as the overall muscle length decreases. Furthermore, if these curves are shifted along the horizontal axis so that the point $(P_0)_l$ lies on the l_0 curve, they are not part of the same curve, indicating that the instantaneous elasticity of an active muscle as a function of load is not just determined by some passive constant element such as the tendons or the compliance of the recording equipment.

In Fig. 2, the stiffness points from six experiments are pooled and compared with the corresponding points of the tension–length relationship for the same muscles. Sarcomere widths were measured using a laser diffraction technique on whole toad sartorii.

The stiffness of the muscle remains at maximum for a short distance below the body length while the tension is falling and then, from a measured sarcomere width of about 1.82 μm, the stiffness and tension curves fall parallel to each other, with the stiffness at a given length always higher than the force. The relationship between the maximum isometric force at a given length and the stiffness at that length is shown in Fig. 3. To a first approximation, these points are linear to about $0.85P_0$ where the first sign of a decline in stiffness is observed. It was difficult to obtain values of stiffness for points below $0.2P_0$ because of the deterioration of the stimulated muscles at very short lengths[5] The stiffness–force curve may have an intercept on the stiffness axis or may fall quite sharply to zero at points below about $0.2P_0$.

A striking feature of our experiments is the similarity of our results to the relationship between metabolic activity and force at different muscle lengths in isometric tetani. Figure 3 shows the relative change in creatine phosphate hydrolysis[6], maintenance heat rate[7] and stiffness with peak tetanic tension in muscles above and below l_0. At long muscle lengths ($>l_0$) there is a very good correlation between the measured stiffness, metabolic activity and peak tetanic tension. At lengths below l_0, the stiffness is more directly related to the creatine phosphate hydrolysis and maintenance of heat rate than to isometric tension Moreover, the fact that the relative metabolic activity and normalised stiffness are consistently higher than the force suggests that there may be some internal force which opposes shortening below l_0. Such a force would naturally
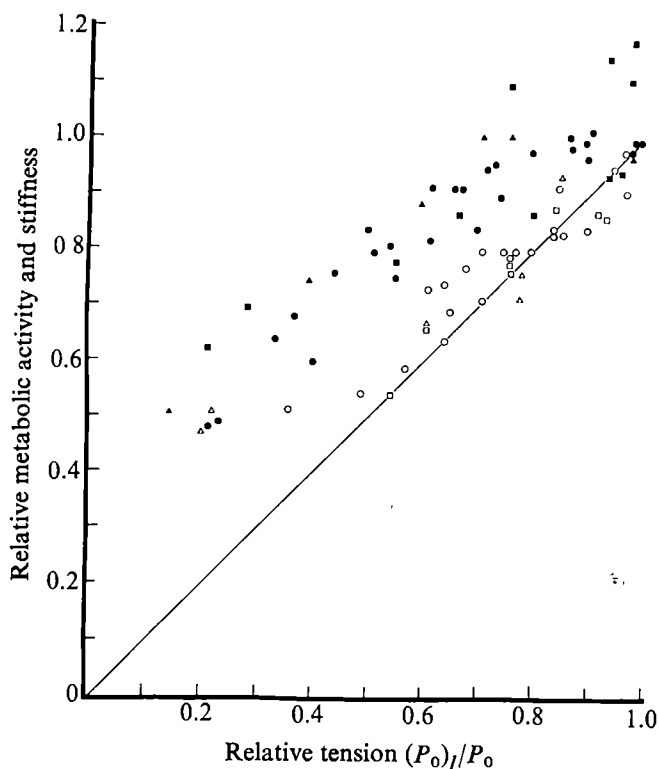
Fig. 3 Relationship between metabolic activity, instantaneous stiffness and maximum tetanic tension at different muscle lengths. Closed symbols, values for lengths below l_0; open symbols, lengths above l_0. All values have been normalised with respect to their maximum value at l_0. ■, □, Maintenance heat rate; ▲, △, creatine phosphate hydrolysis; ●, ○, instantaneous stiffness. Correlation coefficient for fit of open symbols to the line through the origin and the 1, 1 point is $r=0.91$; for closed symbols $r=0.76$.

subtract from the tension produced by the myofilaments. Evidence for such a force has been provided by Gordon *et al.*[8] who have calculated an increase in internal osmotic pressure of 1 kg cm^{-2} in a muscle shortened from 40 to 75% of its initial length. Additional evidence has been provided by Simmons[9] who has shown that single fibres allowed to shorten freely in solutions of increasing tonicity exhibit a definite resistance to shortening at sarcomere lengths of 2.0 μm and between 1.92 and 1.95 μm. This corresponds to the range of sarcomere lengths over which the measured stiffness seems to be constant as the peak isometric tetanic tension declines.

In previous experiments which showed a decrease in the stiffness of contracting skeletal muscle at long lengths[1,10] it seemed possible, though perhaps unlikely, that the thin filaments in the I band might be a major source of muscle compliance. If the elastic behaviour of the active muscle were dominated by the compliance of the I band, then as the sarcomere shortens and the I-band width decreases, the overall stiffness of the muscle should increase dramatically. Our work, showing that, on the contrary, the stiffness of the contracting toad sartorius decreases as the sarcomere length is reduced and as the maximum isometric tetanic tension falls, rules out such a possibility.

This work was supported by the Canadian MRC and the Muscular Dystrophy Association of Canada.

BERNARD H. BRESSLER*

Department of Physiology,
University of Saskatchewan,
Saskatoon, Saskatchewan, Canada

NORMAN F. CLINCH

Department of Physiology,
University of Manitoba,
Winnipeg, Canada

Sulphydryl groups and the quinone receptor in insect olfaction and gustation

UNDERSTANDING of energy-transduction aspects of olfactory messenger–receptor interactions has been lacking[1], until the recent advances[2–6] made using the chemoreceptor protein for quinone messengers, isolated from sensory nerve membranes in the antennae of *Periplaneta americana*[7,8]. Availability of this protein for *in vitro* experiment has filled a void that existed, for several decades, between the behavioural and electrophysiological experimental capabilities in this field. With purified receptor protein available, physicochemical aspects of olfaction and gustation have become amenable to direct experiment. Here we present evidence that messenger naphthoquinones showed marked selectivity for the receptor protein when it was exposed *in vitro* to a saturating 10^{-4} M concentration of each messenger in the presence of the other Triton-solubilised proteins from the insect's antennae. The electrophoretically resolved[4,7,8] receptor-containing band only constituted approximately 13.7% of the protein in the Triton-soluble fraction, but it bound more than 75% of radiolabelled messengers. The other messenger-associated radioactivity was retained at the origin of the gels or was scattered as low level DPMs among the several other resolved bands These findings also confirm earlier experimental results[8] on the selective binding of messenger to the receptor band as resolved in 3.5% acrylamide gels.

The possible involvement of ATPases in this chemoreception by *P. americana* has been considered previously[9], and we now report that when the Triton-soluble proteins from the antennae were pre-incubated with saturating 10^{-4} M ouabain for 15 min before the addition of messenger 2-methyl-^{14}C-1,4-naphthoquinone (menadione), the amount of quinone messenger bound to the receptor was not significantly reduced from that with the control at the 0 01 level of probability Thus, active ouabain-sensitive ATPases are not involved in the primary messenger–receptor interactions in this olfactory and gustatory process.

With regard to reaction sites in the receptor, the sulphydryl reagents, N-ethyl maleimide (NEM) and iodoacetic acid (IAA), at a saturating concentration of 10^{-4} M in the Triton-soluble proteins from the antennae, showed selectivities of binding with the receptor band[4,8] in 3.5% gels that were comparable with those of the messenger naphthoquinones. The receptor-containing band was recently shown[4] to be exceptionally rich in sulphur-containing amino acids, and thus the now reported marked selectivity of the sulphydryl reagents seems reasonable. As a direct indication of the major role of sulphydryls as actual functional groups in the receptor for the messenger naphthoquinones, IAA binding to receptor was competitively reduced by preincubation of receptor with each of four naphthoquinones (Fig. 1). The order of this *in vitro* competitive binding at quinone concentrations of 5×10^{-6} to 10^{-5} M was the same as the order of the relative unchallenged binding of the four naphthoquinones to receptor, repellency or inhibition of feeding as measured in bioassays[2,10], inhibition of chemosensory neurones as

measured by electroantennograms (EAGs)[5], binding to nerve membrane-rich density gradient fractions from antennae[2], shifting of the polarographic half-wave potential ($E_{1/2}$) of the isolated receptor protein[3], and inhibition of ouabain-sensitive ATPases[9]. The completely compatible experimental results from all these levels of examining these messenger–receptor interactions support the final conclusion that sulphydryls are major reaction groups (sites) for the messenger naphthoquinones' selective binding to this receptor protein.

The receptor protein was extracted from adult male *P. americana* in the same manner as detailed previously[3,4,7,8]. The messenger naphthoquinones or sulphydryl reagents, NEM or IAA, as used as the treatment chemical in the selective binding experiments, were added to the preparation during initial tissue maceration. In the competition experiments, the ouabain was added during initial tissue maceration, or the respective messenger naphthoquinone (Fig. 1) was introduced into uniform aliquots of the receptor extract. Each extract was then incubated for 15 min before introduction of the challenger, ^{14}C-menadione or ^{14}C-labelled IAA (Fig. 1) This sequence allowed each initial treatment chemical unchallenged access to the reaction sites in the receptor protein Electrophoretic separation, the staining and quantitation of protein, the slicing of gels, and counting of radioactivity have been described[8].

Messenger naphthoquinones showed a marked selectivity of binding for the electrophoretically resolved band of Triton-soluble protein from the antennae of *P. americana* which we previously termed the quinone receptor. Sulphydryls were proven to be major reaction sites in the receptor. The fact that the primary receptor activity does not require an active ouabain-sensitive ATPase was proven by the failure of a preincubation of receptor with ouabain to alter significantly the binding of messenger naphthoquinone. This finding is compatible with our previous demonstration[9] that messenger menadione does not significantly inhibit ouabain-sensitive ATPases. Thus, any disruption of chemoreception by the ouabain-sensitive ATPase inhibiting properties of naphthoquinone messengers with a significant amount of such activity (for example, juglone, 1,4-naphthoquinone, and lawsone) is strictly attributable to effects at the non-primary levels of this sensory process.

The real test of having identified critical physicochemical

Fig. 1 Average pmol of ^{14}C-labelled IAA subsequently bound in the electrophoresed receptor-containing protein band when 10^{-4} M IAA was challenged for receptor sites by a 15 min preincubation of the receptor with increasing concentrations of each of four messenger 1,4-naphthoquinones
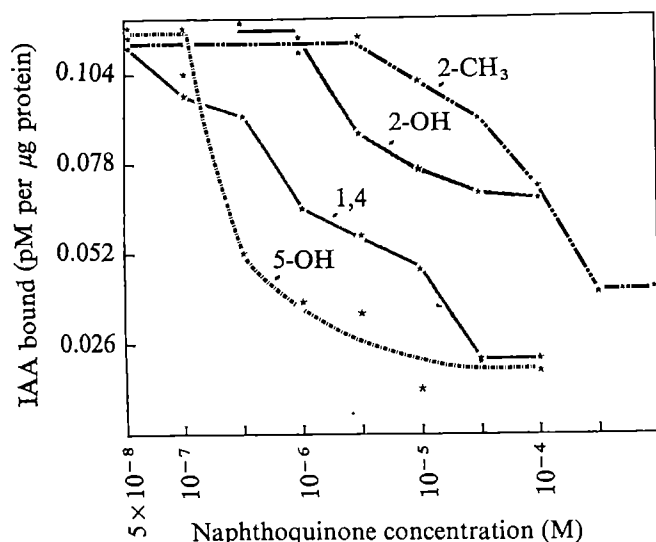
parameters of this chemoreception process is the use of the obtained information as the bases for predictable alteration of the sensitivity of the system in the live animal. This has been accomplished on an extensive scale, and those results are presented elsewhere[6,10].

GEORGE SINGER
JACK M. ROZENTAL
DALE M. NORRIS

246 Russell Laboratories,
University of Wisconsin,
Madison, Wisconsin 53706

1 Kaissling, K. E, in *Handbook of Sensory Physiology*, 4, *Chemical Senses*, 1, *Olfaction*, 518 (Springer, Berlin, 1971).
2 Norris, D. M., Ferkovich, S M, Baker, J. E., Rozental, J. M, and Borg, T. K., *J. Insect Physiol.*, 17, 85–97 (1971).
3 Rozental, J M., and Norris, D. M, *Nature*, 244, 370–371 (1973).
4 Singer, G, Rozental, J. M., and Norris, D. M, *Experientia*, 30, 1384–1385 (1974).
5 Norris, D. M, and Chu, H. M., *J. Insect Physiol.*, 20, 1687–1696 (1974).
6 Rozental, J M, and Norris, D. M., *J. Insect Physiol* (in the press)
7 Ferkovich, S M., and Norris, D. M., *Experientia*, 28, 978–979 (1972).
8 Singer, G., and Norris, D. M., *Experientia*, 29, 1483–1484 (1973).
9 Baker, J. E., and Norris, D. M., *J Insect Physiol.*, 17, 2383–2394 (1971).
10 Rozental, J. M, and Norris, D. M., *Life Sci.* (in the press).

Murine sarcoma virus pseudotypes acquire a determinant specifying N or B tropism from leukaemia virus during rescue

SEVERAL classes of murine leukaemia viruses (MuLV) are recognised according to their ability to induce XC plaque formation with high or low efficiency after infection of various mouse cell lines[1,2]. N- and B-tropic MuLVs, for example, can be distinguished on the basis of their relative ability to infect N-type or B-type mouse cells, the cells themselves differing at a single genetic locus, Fv-1 (ref. 3). Fv-1 restriction is not absolute[3], is dominant over susceptibility in F₁ hybrids[4], and may be mediated by a specific intracellular inhibitor[5,6]. Studies using vesicular stomatitis virus (VSV) pseudotypes made with N- or B-tropic MuLV have shown that Fv-1 restriction occurs after virus penetration[7,8], although the mechanism of inhibition is not known. A clear difference exists between Fv-1 restriction of MuLV and the host range restriction of avian leukaemia virus subgroups, in which host range restriction occurs at the cell surface[9,10]. Another class of MuLVs, NB-tropic, does not seem to be sensitive to Fv-1-mediated restriction[1].

Restriction of N- and B-tropic MuLVs is of interest as an example of host control of exogenous RNA tumour virus infection. Moreover, an understanding of Fv-1-mediated restriction may also shed light on the mechanism of control of endogenous viral genes which are known to exist, and are often unexpressed, in most, if not all, vertebrate cells[11].

Murine sarcoma viruses (MSVs) are a second group of murine RNA tumour viruses distinguishable from MuLV by an ability to induce sarcomas in mice and to transform a variety of cells in culture[12]. Although MSV can transform cells after solitary infection, most of the MSV isolates studied to date are termed 'defective' as they are unable to replicate unless their host cell is, in addition, productively infected with MuLV (or other 'helper' leukaemia virus)[13,14]. Certain helper-specified products seem to be required for the production of infectious MSV particles. The leukaemia virus has been shown to determine the host range[2,15] and antigenic specificity[16,17] of infectious MSV particles, which are, therefore, pseudotypes with respect to these properties. The ability of leukaemia viruses to specify properties of defective MSV has frequently been useful in determining

the function of leukaemia virus gene products. We now report that several isolates of defective MSV acquire N- and B-tropic specificities from their MuLV helper viruses.

Table 1 summarises the results of a series of focus assays using three MSV-Moloney stocks rescued from MSV-transformed BALB/3T3 non-producer cells[13] with either N-, B-, or NB-tropic MuLV. Each MSV stock, in turn, was assayed on normal BALB/3T3 cells (B type)[1,18], on a continuous line of C3H cells (N type)[4,19], and on 3T3FL cells (dually permissive)[20].

As can be seen in Table 1, MSV stocks rescued with either N-tropic or B-tropic MuLV form foci with equal efficiency in 3T3FL cells when assayed in the presence of optimal NB-tropic helper virus[20]. Also, MSV rescued with dual-tropic MuLV is equally efficient at focus formation in all three cell types.

In contrast, the stock of MSV rescued with N-tropic MuLV was almost 200 times as efficient at focus formation on N-type C3H cells as on BALB/3T3, whereas the same MSV isolate rescued with B-tropic MuLV was only 1/20 as efficient at focus formation on C3H cells (Table 1). Thus, there was nearly a 4,000-fold variation in the relative focus-forming ability of virus stocks prepared from this strain of MSV, depending on the tropism of the rescuing helper virus. (A different conclusion was reached by Yoshikura[21], possibly because the MSV focus assay procedures used did not effectively eliminate the contribution of N- or B-tropic MuLV to focus formation by rescued MSV stocks.)

The MSV assays described above and shown in Table 1 were carried out in the presence of optimal amounts of NB-tropic MuLV 'helper' virus[22]. The foci scored in these assays arise as a result of the spread of progeny MSV from transformed cells; in these conditions the possibility of interactions between MuLV 'helper' virus and N- or B-tropic MSV cannot be excluded. Replicate assay plates were therefore included without added helper virus These plates were counted 5 d after infection, and then allowed to incubate for a total of 11–13 d, at which time they were scored a second time for MSV foci. In the extended assay conditions, foci derived from MSV transformation of single cells in the absence of replicating leukaemia or sarcoma virus may be observed[23].

When the three MSV pseudotypes were tested by the longer assay procedure (Table 1), the N- and B-tropic patterns were identical to those observed above with the

same MSV stocks. MSV rescued with N-, B-, or NB-tropic MuLV exhibited the same tropism as its helper leukaemia virus. Thus, restriction of tropic MSV affects its ability to transform cells and is not altered by the presence of replicating NB-tropic MuLV in the cells used for assay. Note that restriction of N-tropic MSV by B-type cells is more pronounced than is restriction of B-tropic MSV in N-type cells, whereas the reverse pattern is observed with N- or B-tropic MuLVs[1] The restriction of B-tropic MSV in N-type cells is especially weak in assays without optimal helper virus, and, although it is a consistent finding, its significance is difficult to assess.

Foci were also counted 5 d after MSV infection in the above experiment Each of the three MSV pseudotypes showed a typical two-hit dose–response curve when assayed in permissive cells in the absence of added helper virus (data not shown)[22].

Table 1 data were obtained by titration of MSV rescued from transformed non-producer BALB/3T3 cells. A similar experiment, using dually permissive S+L- 3T3FL cells as the source of the MSV stocks, yielded nearly identical results. Therefore the Fv-1 restriction exhibited by the cell from which MSV is rescued does not seem to be a major factor in determining MSV tropism.

In addition to the Moloney isolate of MSV described above, we also tested MSV rescued from BALB/S+L cells[20], from K-BALB cells[24], and from a line of Harvey sarcoma virus-transformed 3T3FL cells recently isolated in our laboratory. All of the MSV isolates showed very nearly the same pattern of focus formation as that shown in Table 1 following rescue with N-, B- or NB-tropic MuLVs.

Our data thus show that the infectivity of MSV stocks is subject to Fv-1 restriction. MSV rescued by N- or B-tropic MuLV shows the same tropism as its helper virus, whereas dually tropic MuLV gives rise to dually tropic MSV. Cells which restrict N- or B-tropic MuLVs exhibit the same restriction to corresponding MSV pseudotypes, and the 3T3FL cell line is equally permissive to N-, B- and NB-tropic MuLV stocks as well as to their MSV derivatives (Table 1). As has been observed with N- and B-tropic MuLVs, Fv-1 restriction of MSV pseudotypes is not absolute—some MSV foci are produced in non-permissive cells.

These results indicate that MSV pseudotypes are subject to the same intracellular restriction mechanisms as are N- and B-tropic MuLVs. The tropism of the defective MSV particle is determined by its helper MuLV during the rescue

Table 1 Effect of Fv-1 restriction on the focus-forming activity of tropic MSV pseudotypes

MSV pseudotype	3T3FL (dually permissive)	Assay cell line C3H (N-type)	BALB/3T3 (B type)	Ratio: N/B*
MSV(N)†	4.2×10^3‡	5.6×10^3	3.1×10^1	180/1
	(1.2×10^4)	(9.6×10^3)	(2.5×10^1)	(382/1)
MSV(B)	2.2×10^4	6.4×10^2	1.3×10^4	1/20
	(1.1×10^5)	(2.6×10^3)	(9.3×10^3)	(1/3.6)
MSV(NB)	6.2×10^4	40×10^4	2.7×10^4	1.5/1
	(1.8×10^5)	(1.7×10^5)	(2.7×10^4)	(6.3/1)
Standard§	2.8×10^5	7.4×10^4	5.6×10^4	1.3/1
	(1.7×10^5)	(ND)	(5.5×10^4)	(—)

MSV stocks were prepared following quantitative MuLV superinfection of MSV-Moloney-transformed non-producer BALB/3T3 cells. Rescue procedures were essentially as described previously[28], and virus pools were collected 11 d after superinfection. The N- and B-tropic MuLVs used for MSV rescue were filtered tissue culture supernatants of chronically infected permissive cell lines. Cloned seed stocks of these viruses were kindly provided by Dr J. Hartley of the NIH, Bethesda, Maryland. The IC clonal isolate of NB-tropic Moloney leukaemia virus[29], grown in dually permissive 3T3FL cells, was used in both the rescue experiments and as optimally diluted 'helper' virus in appropriate MSV assays. The tropism of each MuLV stock as well as the Fv-1 restriction of each cell line used was confirmed by XC plaque titration on BALB/3T3 and C3H cells[30]. Assay plates (60 mm) were seeded with 150,000 normal BALB/3T3 (B type), 80,000 C3H (N type) or 80,000 3T3FL (dually permissive) cells. All cells were treated with DEAE-dextran (20 μg ml⁻¹) before infection.
*Ratio of MSV titre in C3H cells to MSV titre in BALB/3T3 cells.
†MSV(N) = MSV rescued with N-tropic MuLV, and so on.
‡Each number represents MSV focus-forming units ml⁻¹ based on an average of two or three assay plates within the countable range. Bracketed numbers are based on foci counted 11–13 d after infection without added helper virus; numbers not in brackets are based on foci counted 5 d after infection in the presence of optimal helper virus.
§A laboratory standard pool of MSV rescued from S+L-3T3FL cells with NB-tropic MuLV was included with each assay.

process; N or B tropism is therefore a phenotypic, helper-dependent characteristic of MSV pseudotypes. Coinfection of newly infected cells with moderate amounts (m.o i.~0.1) of NB-tropic helper virus during an MSV assay does not seem to alter significantly the tropic properties of MSV pseudotypes (Table 1).

The existence of N- or B-tropic MSV pseudotypes has several implications concerning the nature of Fv-1 restriction. First, the fact that MSV acquires N or B tropism from its MuLV helper implies that the determinant present in leukaemia virus particles which specifies N or B tropism is transferable to MSV during the rescue process. Once transferred, this determinant does not become an integral part of the MSV genome. The determinant of N or B tropism may be responsible for either resistance or sensitivity to Fv-1 restriction, and NB-tropic MuLV may contribute either two determinants or none at all to its defective MSV pseudotype during rescue The factor responsible for the SV1-mediated MSV tropism is very likely distinct from the helper-derived envelope glycoprotein(s) responsible for the antigenic specificity and interspecies host range of MSV pseudotypes, as the Fv-1 restriction mechanism acts after virus penetration of resistant cells, whereas envelope glycoproteins are involved primarily in virus adsorption[25]. The determinant of MSV tropism may be analogous to the helper-dependent factor required for MSV transformation[26], found using MSV rescued by certain temperature-sensitive mutants of MuLV.

Second, MSV pseudotypes are restricted in non-permissive cells not only in their ability to replicate, but also in their ability to transform cells (Table 1) Therefore, while restriction of N- and B-tropic viruses in non-permissive cells involves the production of infectious progeny, it also extends to the expression of an MSV gene product which functions in cell transformation, and not, presumably, in leukaemia virus replication. Restriction associated with the Fv-1 locus may thus involve the simultaneous repression of several viral gene products normally produced after integration of viral information into host cell genetic material[27], or restriction may be the result of an inhibition of the integration process itself. Molecular studies with tropic MSV in permissive and non-permissive cells could distinguish between these two possibilities.

We thank Ms C. A. Kindley for assistance. These studies were supported in part by a contract from the Virus Cancer Program of the National Cancer Institute. S. G. was an NIH Visiting Fellow.

Note added in proof: The resistance of BALB/3T3 cells to transformation by MSV (N) can be abrogated by the addition of large amounts of N-tropic MuLV, but not by comparable amounts of B-tropic or NB-tropic MuLV.

ROBERT H BASSIN   BRENDA I. GERWIN
GRACIELA DURAN-TROISE   SYLVIANE GISSELBRECHT*

Viral Leukemia and Lymphoma Branch,
National Cancer Institute,
Bethesda Maryland 20014

ALAN REIN

Litton Bionetics Inc.,
7300 Pearl Street,
Bethesda, Maryland 20014

1 Hartley, J W , lowe, W P , and Huebner, R J , J Virol , 5, 221–225 (1970)
2 Levy, J A., Science, 182, 1151–1153 (1973).
3 Pincus, T , Rowe,W P., and Lilly, F., J exp Med., 133, 1234–1241 (1971).
4 Pincus, T., Hartle:, J. W , and Rowe, W P , J exp. Med , 133, 1219–1233 (1971).
5 Tennant, R W , Nyer, F E , and McGrath, L , Int J. Cancer, 14, 504–513 (1974).
6 Tennant, R W , Schluter, B , Yang, W-K , and Brown, A , Proc natn. Acad Sci U S A , 71, 4241-4245 (1974)
7 Kronniris, T G , Sietro, R , and Fields, B N , Proc natn Acad Sci U.S.A , 70, 2549–2553 (1973)
8 Huang, A. S , Besmei, P , Chu, L , and Baltimore, D., J Virol , 12, 659–662 (1973).
9 Piraino, A , Virology,32, 700–707 (1967)
10 Crittenden, L B , J natn Cancer Inst , 41, 145–153 (1968)
11 Huebner, R. J , and Todaro, G. J , Proc natn Acad. Sci U S A., 64, 1087–1094 (1969).

12 Harvey, J J., and East, J , Int. Rev. exp. Path , 10, 265–360 (1971).
13 Aaronson, S. A., and Rowe, W P., Virology, 42, 9–19 (1970)
14 Bassin, R. H. Tuttle, N , and Fischinger, P. J., Int J. Cancer, 6, 95–107 (1970).
15 Fischinger, P. J., and O'Connor, T. E., Science, 165, (714–719) 1966
16 Huebner, R J., Hartley, J W , Rowe, W P , Lane, W. T., and Capps, W I., Proc. natn Acad. Sci U.S.A , 56, 1164–1169 (1966).
17 Gomard, E , Leclerc, J. C., and Levy, J. P., J natn. Cancer Inst., 50, 955–961 (1973)
18 Aaronson, S. A , and Todaro, G. J , J cell Physiol, 72, 141–148 (1968).
19 Reznikoff, C A., Brankow, D. W , and Heidelberger, C , Cancer Res., 33, 3231–3238 (1973).
20 Gisselbrecht, S , Bassin, R H., Gerwin, B I , and Rein, A , Int J Cancer, 14, 106–113 (1974)
21 Yoshikura, H , J gen. Virol , 19, 321–327 (1973)
22 Hartley, J. W., and Rowe, W. P , Proc. natn Acad. Sci U.S A , 55, 780–786 (1966).
23 Aaronson, S A., Jainchill, J L., and Todaro, G J , Proc. natn. Acad Sci. U S A., 66, 1236–1243 (1970).
24 Aaronson, S A , and Weaver, C. A., J gen Virol , 13, 245–252 (1971)
25 Love, D. N., and Weiss, R A., Virology, 57, 271–278 (1974).
26 Stephenson, J. R., and Aaronson, S. A , Virology, 58, 294–297 (1974).
27 Varmus, H. E., Vogt, P. K., and Bishop, J M., Proc natn. Acad Sci. U S A , 70, 3067–3071 (1973)
28 Peebles, P T., et al., J. Virol., 8, 690-694 (1971).
29 Fischinger, P J , Moore, C. O , and O'Connor, T E , J natn Cancer Inst., 42, 605–622 (1969)
30 Rowe, W. P., Pugh, W E., and Hartley, J. W , Virology, 42, 1136–1139 (1970).

Induction of erythroid leukaemia by Harvey and Kirsten sarcoma viruses

SEVERAL RNA tumour viruses including the Friend[1], Rauscher[2] and Kirsten (MLV-Ki)[3] strains cause erythroid leukaemia in mice. These agents do not transform cells *in vitro*, but provide a helper activity for the replication of defective transforming sarcoma viruses. The Kirsten sarcoma virus (KiSV)[4] was isolated from a rat inoculated with MLV-Ki, whereas the Harvey sarcoma virus (HaSV)[5] was recovered from a rat infected with the non-transforming Moloney leukaemia helper virus (MLV-M)[6]. Both sarcoma viruses contain common rat genetic sequences[7,8].

Stocks of both KiSV and HaSV cause erythroid leukaemia and solid tumours in mice[4,9,10]. The agent responsible for inducing the erythroid leukaemia has not, however, been identified. Recently, clonal KiSV- and HaSV-transformed non-producers, free of associated leukaemia virus, have been isolated *in vitro*[11–13]. We have rescued these defective sarcoma viruses with MLV-M and now show that these transforming viruses cause erythroid leukaemia in mice

Cells were maintained in Dulbecco's modified Eagle's medium containing 4.5 g l^{-1} glucose and 10% calf serum (Colorado Serum Company). The establishment of the non-transformed cell lines, NIH/3T3 (ref. 14), NRK (ref 15), and BALB/c-3T3 (ref. 16), and the isolation of HaSV- and KiSV-transformed non-producer cells[11–13] have been described. A clonal isolate of MLV-M (ref 17) was grown on NIH/3T3 cells. MLV-M was quantitated by its ability to induce syncitia formation in XC cells[18]. About 10^6 non-producer cells were transferred to a Falcon flask (area, 75 cm^2) containing 4 μg ml^{-1} polybrene (Aldrich) and infected 24 h later with 10^7 XC units MLV-M. Infected or mock-infected cells were passaged for 2 weeks. To prepare virus stock, 10^6 cells were transferred to a 75 cm^2 flask; tissue culture fluids were collected 24 h later and stored at −70 °C The *in vitro* assay for the replication of the transforming activity on NIH/3T3 cells has been described[19]. To induce erythroid leukaemia, virus stock was passed through a membrane filter (pore size, 0.45 μm) and 0 1 ml was inoculated intraperitoneally into newborn Swiss mice Three to four weeks later, the animals were killed and autopsied. Spleens were sectioned sagittally and touch preparations were made and stained with the Wright–Giemsa stain or benzidine. The spleens were then fixed[20] and the uncut surface examined for erythroblastic foci[21]. Solid tumours were identified grossly and further characterised after staining with haematoxylin and eosin[20].

MLV-M-infected HaSV- or KiSV-transformed non-producers yielded a virus that transformed NIH/3T3 and caused erythroid leukaemia (Table 1). MLV-M did not transform NIH/3T3 or cause erythroid leukaemia, nor did medium conditioned by the non-producers. Thus, the HaSV and KiSV caused erythroid leukaemia because their transforming

Table 1 Rescue of the transforming erythroid leukaemia–sarcoma viruses

Cell line	Addition of MLV-M	Replication of transforming activity	Erythroid leukaemia	Recovery of transforming virus from spleens
KiSV-NIH	No	−	−	ND
	Yes	+	+	+
KiSV-BALB	No	−	−	ND
	Yes	+	+	+
KiSV-NRK	No	−	−	ND
	Yes	+	+	+
HaSV-NIH	No	−	−	ND
	Yes	+	+	+
NIH/3T3	No	−	−	ND
	Yes	−	−*	−

One day after addition of MLV-M, the infected or mock-infected KiSV-NRK were cocultivated with 10^6 NIH/3T3 cells. Virus stocks were prepared from the medium overlaying these and other cultures as described. To assay for the recovery of transforming virus, spleens were shredded through a wire grid and a 10% cell suspension made in medium containing calf serum. The cells were removed by low speed centrifugation; the supernatant was passed through a 0.45 μm membrane filter and assayed for transforming activity on NIH/3T3 cells[19].

*Some of the mice inoculated with MLV-M developed an erythroid response, which could be noted in the spleen at 1 month of age. This response could be readily differentiated from erythroid leukaemia: the cells matured normally and the predominant nucleated erythroid cell was the orthochromic normoblast; there was no evidence of haemorrhage; and discrete foci were not evident. A similar erythroid response has been noted with the Friend helper virus[26]. Approximately 80% of these mice eventually developed the thymic tumours of Moloney leukaemia 3 months after inoculation.
ND, not determined.

activity could not be separated from their leukaemogenic activity.

A virus recovered from the spleens of the erythroid leukaemic mice transformed NIH/3T3 cells (Table 1). These transformed cells had the typical compact and piled-up appearance of KiSV- or HaSV-transformed NIH/3T3. MLV-M was also present in these spleens and was detected by the XC cell test[18].

Animals with erythroblastic leukaemia had grossly enlarged spleens with many pale, ill-defined erythroblastic foci[21]. These spleens often ruptured causing the death of the animal. More than 30% of the cells in areas of

Fig. 1 Assay of virus stock for the induction of erythroid leukaemia or sarcomas in newborn Swiss mice. Virus stock was prepared by rescuing the transforming agents from HaSV-transformed NIH/3T3 or KiSV-transformed BALB/c-3T3 with MLV-M. Both stocks had a titre of about 10^5 transforming units and 10^6 XC units ml⁻¹. Approximately 20 mice were inoculated with each dilution of 0.45 μm filtered virus stock. □, ■, HaSV (MLV-M); ○, ●, KiSV(MLV-M). □, ○, solid tumour; ■, ●, erythroid leukaemia.

erythroid leukaemia were proerythroblasts or basophilic erythroblasts, and occasional trinucleate forms were seen. There was also an increased number of more mature erythroid cells that gave a positive benzidine stain for haemoglobin. Foci of erythroid leukaemia could also often be identified in the liver.

Some of the animals inoculated with HaSV (MLV-M) or KiSV (MLV-M) also developed solid tumours. These tumours (usually rhabdomyosarcomas or fibrosarcomas) grew slowly and did not kill the host. To compare the frequency of erythroid leukaemia with that of solid tumours, animals were inoculated with serial dilutions of virus stock. At all dilutions that caused disease, the incidence of erythroid leukaemia was greater than that of solid tumours (Fig. 1). Approximately 10^2 transforming units of HaSV or KiSV caused microscopic foci of erythroid leukaemia in 50% of the inoculated animals, while 10 transforming units did not cause erythroid leukaemia. HaSV (MLV-M) and KiSV (MLV-M) caused erythroid leukaemia in Swiss, BALB/c and C57BL/6 newborn mice.

Four defective type C viruses with transforming activity for 3T3 (Abelson virus[19,21], Moloney sarcoma virus[11,22], HaSV and KiSV) have been isolated from rodents. One of these agents, the Abelson virus, causes a lymphoid leukaemia of B or null cell origin[23,24], whereas HaSV and KiSV each cause erythroid leukaemia. The transforming activity of these three defective viruses is probably responsible for affecting the growth control of haematopoietic cells to induce leukaemia. Defective transforming viruses recovered from other species may also cause leukaemia.

CHARLES D. SCHER
Hematology and Oncology Division,
Children's Hospital Medical Centre and Sidney
Farber Cancer Center,
Department of Pediatrics, Harvard Medical School,
Boston, Massachusetts 02115

EDWARD M. SCOLNICK
Viral Leukemia and Lymphoma Branch,
National Cancer Institute, Bethesda, Maryland 20014

RICHARD SIEGLER
Martin Luther King, Jr Hospital,
Los Angeles, California 90059

1 Friend, C , *J. exp. Med* , 105, 307–318 (1956).
2 Rauscher, F. J., *J. natn. Cancer Inst.*, 29, 515–543 (1962).
3 Kirsten, W. H , Mayer, L. A., Wollmann, R. L , and Pierce, M/ l., *J. natn. Cancer Inst.*, 38, 117–139 (1967).
4 Kirsten, W. H., and Mayer, L. A., *J. natn. Cancer Inst.*, 39, 311–335 (1967).
5 Harvey, J. J , *Nature*, 204, 1104–1105 (1964)
6 Dunn, T B , Moloney, J. B., Green, A W., and Arnold, B , *J natn. Cancer Inst.*, 26, 188–221 (1961).
7 Scolnick, E. M., Maryak, J M., and Parks, W P , *J. Virol* , 14, 1435–1444 (1974).
8 Tsuchida, N., Gilden, R. V., and Hatanaka, M., *Proc natn Acad Sci. U.S.A.*, 71, 4503–4507 (1974)
9 Chesterman, F. C , Harvey, J. J., Dourmashkin, R. R., and Salama.n, M H., *Cancer Res.*, 26, 1759–1768 (1966)
10 Bassin, R. H., Simons, P J., Chesterman, F. C., and Harvey, J J., *Int. J. Cancer*, 3, 265–272 (1968).
11 Aaronson, S. A., and Rowe, W P., *Virology*, 42, 9–19 (1970).
12 Benveniste, R. E., and Scolnick, E. M , *Virology*, 51, 370–381 (1973)
13 Scolnick, E. M , and Parks, W. P., *J. Virol.*, 13, 1211–1219 (1974)
14 Jainchill, J L , Aaronson, S. A., and Todaro, G. J , *J. Virol.*, 4, 549–553 (1969).
15 Duc-Nguyen, H , Rosenblum, E. N , and Zeigel, R. F., *J. Bact* , 92, 1133–1138 (1966).
16 Aaronson, S. A., and Todaro, G. J., *J Cell Physiol* , 72, 141–148, (1968).
17 Fan, H., and Paskind, M , *J Virol* , 14, 421–429 (1974).
18 Rowe, W. P., Pugh, W E., and Hartley, J. W., *Virology*, 42, 1136–1139 (1970).
19 Scher, C. D., and Siegler, R , *Nature*, 253, 729–731 (1975)
20 Siegler, R., Zajdel, S , and Lane, I , *J natn. Cancer Inst*, 48, 189–218 (1972).
21 Abelson, H. T., and Rabstein, L. S , *Cancer Res.*, 30, 2213–2222 (1970)
22 Moloney, J B , *Natn Cancer Inst Monogr* , 22, 139–142 (1966)
23 Sklar, M D , Shevach, E. M., Green, I., and Potter, M., *Nature*, 253, 550–552 (1975).
24 Rosenberg, N., Baltimore, D , and Scher, C. D., *Proc. natn. Acad. Sci. U.S.A.*, 72, 1932–1936 (1975).
25 Steeves, R. A , Eckner, R. J , Bennett, M., Mirand E. A., and Trudel, P. J , *J. natn. Cancer Inst.*, 46, 1209–1216 (1971).

Transcriptional events during early phase of germination of rice embryos

GERMINATION transfers a metabolically inert embryo into an active state of growth and development. The presence of conserved mRNAs has been demonstrated in different species of eggs and seeds[1-4]. In rice embryos, germination was shown to be independent of the synthesis of RNA up to 18–24 h after the start of imbibition[5], although RNA synthesis was detected as early as 9 h after the start of imbibition. In this report, the sequence of the transcriptional events taking place during the early phase of the germination of rice embryos are presented.

Rice seeds (*Oryza sativa* L.IR-8) were germinated for different periods[5], the embryos were excised, incubated with the radioactive precursor, homogenised, and the ribosomes were separated by sucrose density gradient centrifugation[6]. The amounts of ribosomes, ^{32}P-RNA and ^{14}C-protein associated with monosomes at different stages of germination are shown in Table 1. The increase in the radioactivities of ^{14}C-amino acids and ^{32}P-orthophosphate associated with monosomes of embryos germinated for 12–24 h was reflected in the twofold increase in the amount of monosomes at 24 h. This indicated the synthesis of ribosomal components and maturation of ribosomes by 24 h of germination. The incorporation of ^{32}P-orthophosphate at 6 h was negligible, whereas it increased by sevenfold by 12 h of germination.

The ^{32}P-RNA isolated from the microsomes of embryos excised from seeds germinated for 12 and 24 h in presence of ^{32}P-orthophosphate were analysed by 2.6% polyacrylamide gel electrophoresis[7] to investigate the synthesis of rRNA (Fig. 1). Both the 12 and 24 h samples showed the two rRNA peaks (I and II) indicating synthesis. These results imply that the rRNAs were synthesised in the early phase (0–12 h) and were retained during the entire experimental period (24 h). No significant incorporation of ^{32}P-orthophosphate into microsomes could be detected,

Fig. 1 Analysis of ^{32}P-RNA from microsomes by SDS-polyacrylamide gel extrophoresis. ^{32}P-RNA isolated from microsomal fraction of embryos excised from seeds grown in ^{32}P-orthophosphate for 12 (●) or 24 h (■) was dissolved in electrophoresis buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, and 0.2% SDS adjusted to pH 7.8) and 0.1 ml (46,000 c.p m.) was applied on to 2 6% gel and subjected to electrophoresis at 5 mA per tube for 75 min. The gel was dried between filter paper folds, cut into 1 mm thick strips with the paper and counted. ^{14}C-labelled RNA from *Escherichia coli* was used as standard.
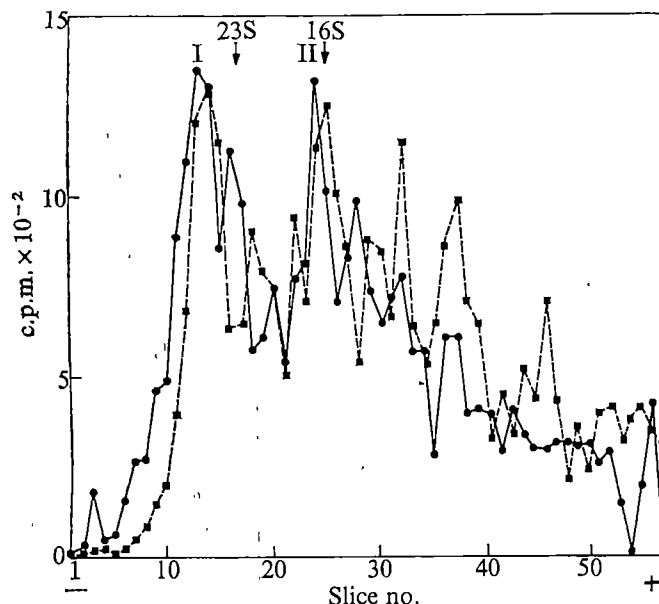
Table 1 Quantity of ribosomes, ^{32}P-RNA and ^{14}C-protein at different stages of germination

Germination (h)	Ribosome (A_{260})	c.p.m. ^{32}P-RNA	c.p.m. ^{14}C-Protein
6	0.30	1,800	9,200
12	0.36	12,500	10,900
24	0.63	44,200	—

Embryos (100) at different stages of germination were homogenised in 3.5 ml 25 mM Tris HCl, pH 7.4, 10 mM MgCl₂, 5 mM β-mercaptoethanol and 0.25 M sucrose with sand in a mortar at 4 °C, centrifuged at 2,500g for 5 min; the supernatant fraction was further centrifuged at 12,000g for 15 min. Supernatant fraction (1 ml) was layered on 27.2 ml 15–30% sucrose gradient and spun at 25,000 r.p.m. in a SW-25.1 rotor for 3 h at 4 °C. Tubes were punctured and fractions of 50 drops were collected. Quantity of ribosomes was estimated by absorbance at 260 nm. For incorporation studies, the embryos were incubated in 2.5 ml 0.1 M Tris-HCl pH 7.8, 1% glucose and 50 μg ml⁻¹ chloramphenicol in presence of ^{32}P-orthophosphate (60 μCi) or ^{14}C-algal protein hydrolysate (10 μCi) at 30 °C for 2 h and the ribosomes were separated as before. Each fraction was made 10% with respect to TCA and the precipitate was washed free of lipids on glass fibre filters and counted[5]. Radioactivity under monosome peak represented ^{32}P-labelled RNAs or ^{14}C-labelled proteins associated with ribosomes.

however, when the seeds were germinated for 6 h in presence of ^{32}P-orthophosphate. This observation and the data presented in the Table 1 indicate that rRNA appears in the cytoplasm between 6 and 12 h of germination. In wheat embryos the mature ribosomes have been detected only after 6 h of germination[8].

Although the 24 h labelled ^{32}P-RNA isolated from microsomes also includes the RNA synthesised up to 12 h, there are differences in the two patterns (Fig. 1). Some new peaks of radioactivity (<16S) did appear by 24 h of germination, indicating the synthesis of a new species of RNA.

The base compositions of ^{32}P-RNAs isolated from embryos labelled for 1 h at 12, 18 and 24 h of germination are given in Table 2. The G+C/A+U ratio of 1.39 of the pulse-labelled RNA at 12 h of germination indicated the predominant synthesis of rRNA during 0–12 h. RNAs synthesised during the later periods have G+C/A+U ratios less than one, indicating their DNA-like nature. The synthesis of RNAs rich in G+U has been reported during the germination of wheat embryos[9]. The base composition of the pulse-labelled RNAs in rice was identical with the conserved mRNA in embryos[4] of wheat seeds and RNAs synthesised during germination[9]. rRNAs are synthesised in rice embryos before the synthesis of mRNA and may have a role in the transport of the mRNAs from the nucleus to cytoplasm[10,11]. The synthesis of mRNA itself takes place after the first cycle of DNA synthesis, the latter being maximum at 12–18 h (unpublished). Analysis of the postmicrosomal supernatant ^{32}P-RNA on 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel showed the incorpora-

Table 2 Base composition of pulse-labelled RNAs at different stages of germination

Germination (h)	% Total GMP	UMP	AMP	CMP	G+C / A+U
12	35 0	21.5	21.1	21.2	1.39
18	25.3	26.7	25.3	22.5	0.94
24	29.9	33.8	18.9	17.9	0.91

Embryos (500) at different stages of germination were labelled for 1 h with ^{32}P-orthophosphate (160 μCi) in 3.5 ml incubation medium (Table 1), homogenised in 6 ml 10 mM Tris-HCl, pH 7.8, 20 mM KCl and 10 mM MgCl₂ and 0.1 M Na₂HPO₄ Homogenate was centrifuged at 1,500g for 10 min and ^{32}P-RNA was isolated from the supernatant fraction[8]. RNA was hydrolysed and the nucleotides were separated by electrophoresis on Whatman no. 1 paper at 2,000V for 105 min in pyridine acetate buffer, pH 3.5, according to the method of Sebrings[12]. Nucleotides were identified by using internal standards and quantitated by the amounts of radioactivity.
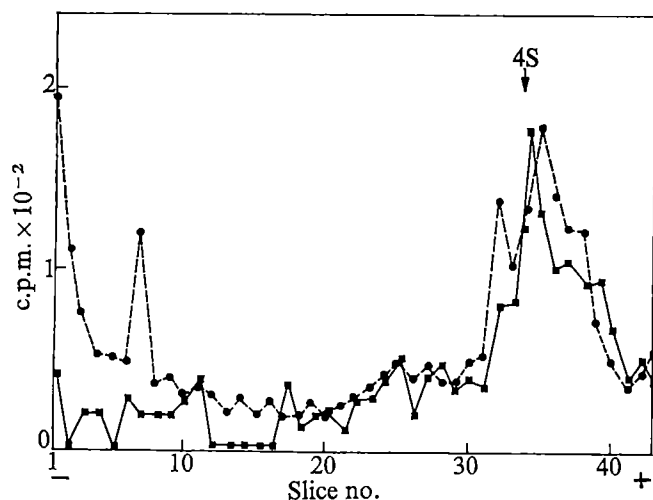
Fig. 2 Analysis of ^{32}P-RNA from postmicrosomal supernatant fraction by SDS-polyacrylamide gel electrophoresis. ^{32}P-RNA from postmicrosomal fraction (■, 6 h; ●, 12 h) was isolated, dissolved in electrophoresis buffer (Fig. 1) and 0.1 ml (2,400 c.p.m.) was applied on 10% gel and subjected to electrophoresis at 5 mA per tube for 3.5 h. Gels were cut into 2 mm thick slices, dried on filter paper disks and counted. ^{35}S-labelled tRNA from *Pseudomonas aeroginosa* was used as standard.

tion of ^{32}P-orthophosphate into 4S RNA as early as 6 h of germination (Fig. 2). This may either represent the synthesis and/or the turnover of CCA ends of tRNAs.

From the data presented above and from the published results[5], commencement of the synthesis of various macromolecules during the early phase of the germination of rice embryos are represented as:

Protein	tRNA	rRNA DNA	mRNA Maturation of ribosome

0————0.5————6————12————24

Time of germination (h)
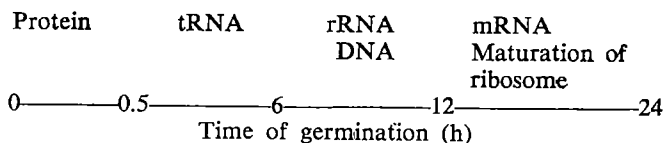
This scheme is at variance with that reported by Dobrzanska *et al.*[9], in which the mRNA synthesis takes place from the beginning of the germination of wheat embryos after presoaking the seeds for 8 h at 2 °C. When wheat seeds were germinated without presoaking, however, synthesis of rRNAs was found to be the earliest transcriptional event[8].

<div style="text-align:right">

S. P. Bhat
J. D. Padayatty
</div>

*Department of Biochemistry,
Indian Institute of Science,
Bangalore 560 012, India*

1 Gross, P. R., in *Current Topics in Developmental Biology*, 2 (edit. by Moscona' A A., and Monroy, A.), 1–46 (Academic, New York, 1967).
2 Nemer, M., in *Progress in Nucleic Acid Research and Molecular Biology*, 7 (edit. by Davidson, J. N , and Cohn, W. E), 243–301 (Academic, New York, 1967).
3 Waters, L. C , and Dure, L. S., *J. molec. Biol.*, 19, 1–27 (1966).
4 Schultz, G. A., Chen, D , and Katchalski, E., *J. molec. Biol.*, 66, 379–390 (1972).
5 Bhat, S P., and Padayatty, J. D , *Ind. J Biochem Biophys* , 11, 47–50 (1974)
6 Padayatty, J. D., and Rolfe, R., *Biochemistry*, 7, 305–316 (1968).
7 Mirault, M. E., and Scherrer, K., *Eur. J Biochem.*, 23, 372–86 (1971)
8 Chen, D., Schultz, G., and Katchalski, E., *Nature new Biol* , 231, 69–72 (1971).
9 Dobrzanska, M , Tomaszewski, M., Grzolezak, Z., Rejman, E., and Buchowicz, J., *Nature*, 244, 507–09 (1973).
10 Bretani, R., Pere, C. S , Oda, C. H., and Wang, L., in *Gene Expression and its Regulation* (edit. by Kenney, F. T., Humkalo, B. A., Favelukes, G., and August, J. T), 169–78 (Plenum, New York and London, 1972).
11 Sirlin, J. L., *Biology of RNA* (Academic, New York and London, 1972).
12 Sebrings, E. D., in *Fundamental Techniques of Virology* (edit. by Habel, K., and Salzaman, N P.), 464–466 (Academic, New York and London, 1969).

Polyribosome formation in early wheat embryo germination independent of either transcription or polyadenylation

EARLY germination of seed embryos is characterised by a rapid increase in polyribosome content and rate of protein synthesis[1,2], and there is evidence[3-8] that these events are a consequence of the mobilisation of preformed messenger RNA[9]. We observed that new mRNA synthesis could be measured at this time of rapid polyribosome formation[10]. Using cordycepin and α-amanitin, inhibitors of this mRNA synthesis, we have now confirmed that preformed mRNA has an important role in early germination, and have found

Sedimentation ⟶
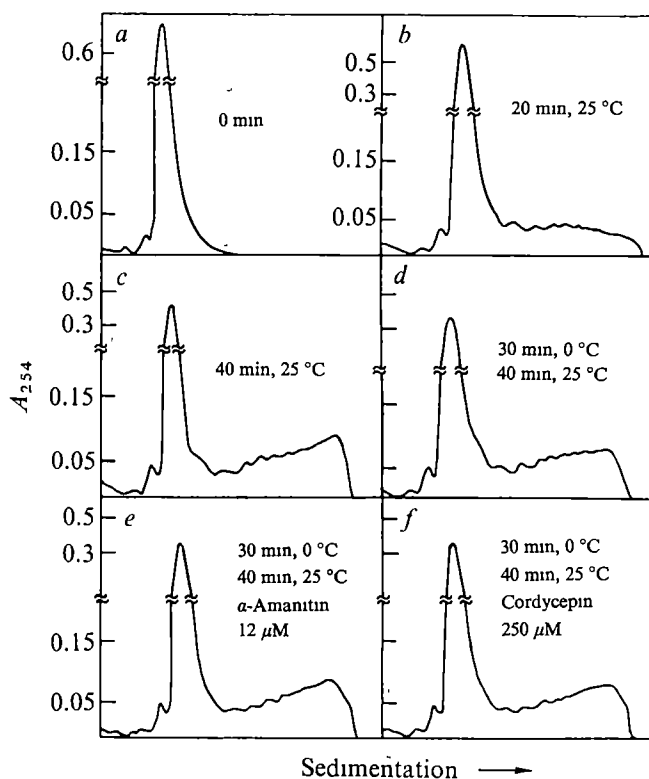
Fig. 1 Polyribosome formation during early germination of wheat embryos and the effect of RNA Inhibitors. Embryos (125 mg) imbibed in 1.6 ml water either directly at 25 °C (*a*, 0 min; *b*, 20 min; *c*, 40 min) or at 1 °C for 30 min followed by 40 min at 25 °C (*d*, control; *e*, 12 µM α-amanitin, *f*, 250 µM cordycepin) were frozen in dry ice. After grinding with a mortar and pestle cooled in dry ice, the "dry ice powder" of the cells was homogenised in a Duall glass homogeniser with 7 ml of isolation media (0.25 M sucrose, 20 mM KCl, 2 mM MgCl₂, 40 mM Tris-HCl pH 7.6, 5 mM mercaptoethanol), cleared by centrifugation (23,000g for 10 min), layered over 2 ml of a cushion of 1.8 M sucrose, 20 mM KCl, 2 mM MgCl₂, 40 mM Tris-HCl pH 7.6, 5 mM mercaptoethanol, and centrifuged for 60 min at 150,000g in a Spinco Ti 50 rotor. (The isolation through the cushion removes some of the monosomes from the ribosome pellet[11] Nevertheless, it is particularly useful both in excluding ribonucleoprotein particles of non-polysomal origin and in increasing the accuracy of the analysis of the change in polyribosome content.) The resulting pellet was suspended in 0.8 ml of 20 mM KCl, 1 mM MgCl₂, 40 mM Tris-HCl pH 7.6, 1 mM dithiothreitol and clarified by centrifugation for 10 min at 23,000g. A volume containing 150 µg RNA was layered on a 15–38% linear sucrose gradient over a 1 5 M sucrose cushion (both gradient and cushion solutions contained 20 mM KCl, 5 mM MgCl₂, 50 mM Tris-HCl pH 7.6, 1 mM dithiothreitol) and centrifuged for 45 min at 230,000g in a SW50 1 rotor. The gradients were fractionated using a modified ISCO density gradient fractionator with continual monitoring of absorbance at 254 nm. The prominent peak of absorbance is that of 80S ribosomes The polyribosome content of the six preparations shown are 3, 22, 55, 46, 42, and 44%, respectively.

Table 1 Effect of cordycepin and α-amanitin on uridine incorporation into RNA of ribosomes during rapid polyribosome formation

Uptake × 10⁸ (c.p m. per 250 mg embryo)	Acid-insoluble radioactivity (c.p.m./mg RNA)	Inhibition (%)
2.6	6,592	
+cordycepin (250 µM)	3.2	1,736	73
+ α-amanitin (12 µM)	2.7	471	93

Embryos (125 mg) preimbibed either water, cordycepin (250 µM) or α-amanitin (12 µM) for 30 min at 0 °C and were then transferred to a similar solution containing either water or the inhibitors, and ³H-uridine (150 µCi) for 40 min at 25 °C. Two batches of embryos were combined; ribosomes were isolated as in Fig. 1; RNA was extracted as in Table 3, and the acid-insoluble radioactivity was determined.

that polyribosome formation at this time is independent of both transcription and of polyadenylation.

Figure 1a-c shows a time course of polyribosome formation in wheat embryos exposed to water at 25 °C. At 20 min considerable mRNA was found in polyribosomes, and by 40 min polyribosomes of maximal size predominated. With some preparations there was no further increase in poly-

tial inhibition of the ultimate increase in protein synthetic capacity. Nevertheless, the time course of polyribosome formation (Fig. 1d) was identical to that at 25 °C. In the presence of either cordycepin (250 µM) or α-amanitin (12 µM), polyribosome formation was unaffected (Fig. 1e and f) Table 1 shows that RNA synthesis during the period of rapid polyribosome formation was inhibited about 70% by cordycepin and more than 90% by α-amanitin. Thus early polyribosome formation is essentially independent of RNA synthesis.

The effectiveness of the inhibitors made possible a test of the involvement of polyadenylation in the recruitment of preformed mRNA, an idea first suggested for the fertilised sea urchin egg[12,13], and recently advanced in studies of enzyme formation in germinating cotton seed cotyledons[14]. We compared the relative incorporation of ³H-uridine and ¹⁴C-adenosine in the presence and absence of the inhibitors. Both these precursors can be expected to label the internal transcribed component of the mRNA, while only adenosine labels the poly(A) tract at the 3′ terminus. If polyadenylation of preformed mRNA were a prerequisite for polyribosome formation, it would not be inhibited by cordycepin or α-amanitin (as neither affects polyribosome formation, Fig. 1), and the process would be revealed by a substantial retention of adenosine incorporation, particularly in relation to that of uridine.*

In practice, the labelled RNA was fractionated into

Table 2 Relative inhibition by cordycepin and α-amanitin of uridine and adenosine incorporation into mRNA

	³H-Uridine		¹⁴C-Adenosine	
	Acid-insoluble Radioactivity (c.p.m./mg RNA)	Inhibition	Acid-insoluble Radioactivity (c.p.m /mg RNA)	Inhibition
poly(A)−	1,263	—	934	—
+ cordycepin (250 µM)	635	50%	657	30%
+ α amanitin (12 µM)	280	78%	245	74%
poly(A)+	3,435	—	2,615	—
+ cordycepin (250 µM)	592	83%	480	82%
+ α amanitin (12 µM)	131	96%	194	93%

Embryos imbibed and were incubated as in Table 1 except that the incubation medium contained ³H-uridine (150 µCi) and ¹⁴C-adenosine (15 µCi). The RNA extracted from the ribosomes was fractionated on oligo(dT)–cellulose columns[16] into non-adsorbing (poly(A)−) and adsorbing (poly(A)+) components. Each fraction was precipitated with 5% trichloroacetic acid, collected on glass fibre filters and counted.

ribosome content for up to 5 h of germination whereas with other preparations we found increases of up to 15% In preliminary trials with inhibitors, we found that RNA synthesis could be inhibited maximally if the embryos had imbibed the inhibitors at 0 °C. In these conditions (30 min at 0 °C) no polyribosomes were formed. Furthermore, no RNA was synthesised during this period. Preimbibition in the cold caused some diminution of polyribosome formation during subsequent exposure to 25 °C (compare percentage polyribosomes of Fig. 1c and d), as well as a par-

poly(A)− and poly(A)+RNA, the latter providing an enrichment of radioactivity incorporated into mRNA. The data obtained (Table 2) show that the presence of the inhibitors, both of which effectively suppress incorporation into mRNA, does not cause a difference in the relative incorporation of the two precursors. We therefore conclude either that there is no significant polyadenylation of the preformed mRNA, or that the inhibitors are as effective in suppressing poly(A) synthesis as they are in preventing transcription The latter alternative would allow polyadeny-

*This expectation is valid only if the amount of preformed mRNA entering the polyribosomes is at least of the same order of magnitude as that synthesised in the period of analysis. The RNA content of polysomes after 40 min, as determined from changes in absorbance (Fig. 1), is 100 µg per 125 mg of embryo. Based on an average molecular weight of 6 × 10⁵ for mRNA and an mRNA concentration of 2% in polyribosomal RNA, this represents a net increase of 3.3 pmol of mRNA The adenosine incorporation into mRNA (Table 2) was 750 c p m per 125 mg embryo. The uptake of 1 × 10⁶ c p m. ¹⁴C-adenosine into the trichloroacetic acid soluble pool (data from the experiment of Table 2). and the ATP content of 150 nmol (ref. 15 and unpublished observations of Cheung. C. P), both per 125 mg of embryo, together with unpublished observations (Obendorf,

R. A.) showing that within 30 min of exposure, 45% of radioactive adenosine taken up by embryos is in ATP, enable the determination of the specific activity of the pool ATP as 3 c p m. pmol⁻¹. Again, based on a molecular weight of 6 × 10⁵ for mRNA and a tract of 100 adenosine residues (molecular weight 30,000) at the 3′ terminus, we have a total of 575 pmol adenosine per pmol of mRNA. The incorporation data calculate to 250 nmol of adenosine incorporated or a net synthesis of 0.4 pmol of mRNA. This value per se suggests that new synthesis could not account for the mRNA mobilised into polyribosomes. It clearly establishes that polyadenylation of preformed mRNA (in the presence of the inhibitors) would be easily detected, if it occurred.

Table 3 Poly(A) content of dry and imbibed wheat embryos

	³H-poly(U) hybridised (ng)	
μg RNA	Dry	Imbibed
6	4	4
11	13	9
28	32	24
55	45	46

Samples of dry and 40-min imbibed embryos (125 mg) were converted into dry ice powders. The powder was suspended in 3 0 ml of extraction solution (0.1 M sodium acetate, 0.1 M NaCl, 50 mM Tris-HCl, pH 8.2, 10 mM EDTA, pH 7 2), made to 0.5% with SDS and mixed with 5 ml of phenol-chloroform (1 : 1, v/v)[17]. The mixture was homogenised for 2 min in a Teflon motor driven homogeniser and centrifuged at 23,000g for 10 min. The aqueous phase was removed and kept on ice, while the phenol phase and the interphase were re-extracted with 3.0 ml of extraction solution at 50 °C After centrifugation, the aqueous phase and the interphase were combined with the previous aqueous phase, and re-extracted with 5 ml of phenol-chloroform for 10 min on a rotary shaker at room temperature. After centrifugation, the interphase was discarded and the aqueous phase was extracted again with 5 ml of phenol-chloroform. The aqueous phase was then mixed with 2 volumes of ethanol and kept overnight at −20 °C. The precipitate was washed twice with 3.0 ml of 3.3 M sodium acetate, pH 6 0 (ref. 18), once with 70% ethanol, 0.1 M potassium acetate, vacuum dried and dissolved in 50 mM potassium acetate, 10 mM Tris-HCl, pH 7.6. Aliquots, as indicated, were taken for determination of ³H-poly(U) hybridisation[19]. The yields of total RNA were 2.5 mg per sample and were similar for both the dry and imbibed samples. The data for poly(U) hybridisation are corrected for a background of 1 ng (22 c p m.) hybridised in the absence of exogenous RNA.

lation of preformed mRNA to occur in early germination but it would not be obligatory to the recruitment of the preformed mRNA. In either event, polyadenylation is clearly not a prerequisite for polyribosome formation. Finally, as a direct test for the occurrence of polyadenylation, we have analysed total RNA of both dry embryos and those that had imbibed for 40 min Table 3 shows that the capacity of the RNAs to hybridise ³H-poly(U) were quite similar. Since the RNA content of both types of embryo was comparable (see legend to Table 3) we conclude that the poly(A) content of embryos either dry or after imbibition is similar, and that the quantity of newly synthesised poly(A)-containing mRNA is negligible in comparison with that pre-existent in the embryo. The mechanism by which the preformed mRNA is recruited into the translational system remains to be investigated.

We thank Dr. T. Wieland for α-amanitin. This research was supported by grants from the US Public Health service, the NIH, and the NSF; and by an appropriation from the Commonwealth of Pennsylvania

SARA SPIEGEL
ABRAHAM MARCUS

The Institute for Cancer Research,
The Fox Chase Cancer Center,
Philadelphia, Pennsylvania, 19111

1 Marcus, A , *Symp Soc exp Biol.*, 23, 143 (1969)
2 Walbot, V., *Devl Biol.*, 26, 369 (1971)
3 Chen, D., Sarid, S , and Katchalski, E., *Proc natn. Acad Sci U S A* , 60, 902 (1968).
4 Waters, L. C., and Dure, L S , *J. molec Biol* , 19, 1 (1966)
5 Walton, D. C , and Soofi, G. S , *Plant Cell Physiol.*, 10, 307 (1969).
6 Chen, D., and Osborne, D. J , *Nature*, 226, 1157 (1970)
7 Walbot, V , *Planta*, 108, 161 (1972).
8 Weeks, D P., and Marcus, A., *Biochim. Biophys Acta*, 232, 671 (1971).
9 Mayer, A. M , and Shain, Y , *A Rev Pl Physiol.*, 25, 167 (1974)
10 Spiegel, S., Obendorf, R. A , and Marcus, A , *Pl Physiol , Lancaster* (in the press)
11 Leaver, C. J., and Dyer, J. A , *Biochem J* , 144, 165 (1974)
12 Slater, I., Gillespie, D., and Slater, D W., *Proc. natn Acad Sci U S A* , 70, 406 (1973)
13 Wilt, F. H , *Proc. natn Acad Sci U.S A* , 70, 2345 (1973)
14 Walbot, V , Capedevila, A , and Dure, L. S , *Biochem. Biophys Res Commun* , 60, 103 (1974).
15 Obendorf, R L , and Marcus, A , *Pl. Physiol , Lancaster*, 53, 779 (1974).
16 Aviv, H , and Leder, P , *Proc. natn Acad Sci U S A.*, 69, 1408 (1972).
17 Perry, R. P., LaTorre, D. E , Kelley, D E , and Greenberg, J. R , *Biochim Biophys. Acta*, 262, 220 (1972).
18 Kirby, K S , *Biochem. J.*, 96, 266 (1965).
19 Rosbash, M., and Ford, P. J , *J. molec. Biol.*, 85, 87 (1974).

Fluoride concentrations in developing enamel

THERE is at present great interest in the effects of fluoride on the skeletal and dental tissues, owing to its considerable beneficial effect on dental health[1], its potential use in the treatment of bone disease[2,3] and, somewhat paradoxically, its capacity to cause dental and skeletal fluorosis[4,5]

The effect of fluoride on teeth has been well documented and particular attention has been paid to its distribution in dental enamel The relatively high concentration of fluoride in the enamel surface[6,7] has been regarded by many as a protective barrier against carious attack. Considerable amounts of fluoride are present in the enamel before the tooth erupts into the mouth[8] and are probably acquired during the period of tooth development when, as indicated by ^{18}F studies[9,10], fluoride ions are rapidly incorporated by the mineralising tissue. Fluoride exerts its effect on the caries susceptibility of the teeth[11] particularly during this period and it is only during the period of development that fluoride can bring about the changes associated with dental fluorosis[12]. In spite of the obvious concern with this period of development, because of technical difficulties with microsampling and analysis, little or nothing is known about the concentrations of fluoride in developing enamel or in any other calcified tissue during its period of mineralisation.

Using recently developed micro-analytical techniques[13] we therefore attempted to determine the pattern of fluoride distribution in the developing enamel of the continuously growing rat incisor; this was chosen because all stages of mineralisation can be seen in a single tooth A preliminary study[14] indicated that in normal animals the concentration of fluoride in the forming enamel decreased as the tissue matured This was somewhat surprising in view of the many statements in the literature that fluoride concentrations in mineralised bone, dentine and enamel tend to increase with time[15-17].

Subsequent work has confirmed our preliminary finding. Wistar rats reared on a stock diet (Oxoid rat cake, fluoride concentration 10–14 p p.m. per dry weight) were killed at about 250 g body weight, the lower incisors removed and a series of about 20 enamel particles weighing 10–100 μg dissected from each tooth. These particles of enamel formed a series representing the different stages of development from partially mineralised tissue near to the root apex to the highly mineralised enamel of the maturing region (Fig 1)[18]. Each enamel specimen was ashed and analysed for fluoride, using the Orion electrode[19], and for phosphorus, using the method of Chen, Toribara and Warner[20] Figure 2 shows the variations in fluoride con-

Fig. 1 Diagram of rat incisor, indicating different stages of enamel development and regions from which enamel samples were taken
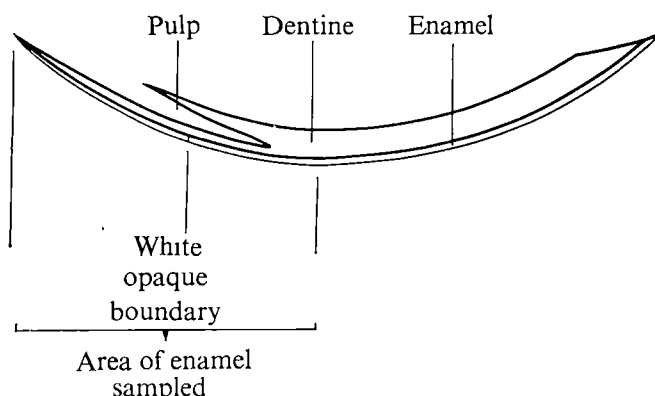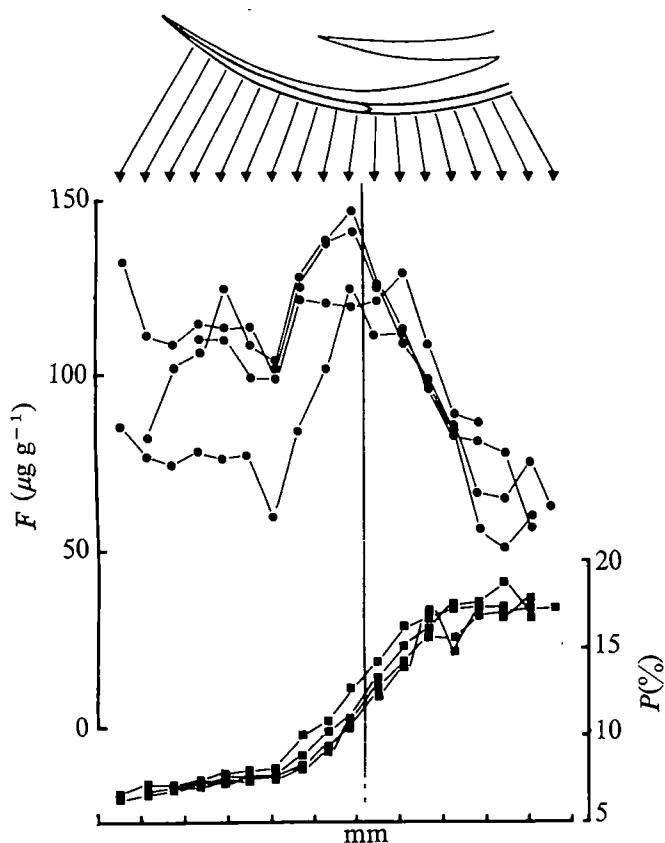
Fig. 2 Distribution of fluoride and phosphorus along the developing enamel of the continuously growing rat incisor (dry weight). The part of the tooth shown and analysed corresponds to the developing part of the root as indicated on the left-hand side of Fig. 1.

centrations and phosphorus concentrations in the developing rat enamel per dry weight tissue. The fluoride concentration was higher in partially mineralised than in the more highly mineralised maturing enamel. The concentration in the partially mineralised enamel was not constant, however. At or just before the stage of development in which the enamel began to mineralise rapidly, fluoride concentrations were particularly high. The concentration fell rapidly as the mineral content of the tissue approached that of mature enamel.

Since information about teeth of limited growth would be more directly relevant to the situation in the human dentition, we carried out similar determinations on enamel dissected from the developing incisors of bovine foetuses. The pattern of fluoride distribution along the enamel of each individual tooth was considerably different from that of the rat incisor This was to be expected, since the enamel of each bovine tooth was at a different stage of development, depending on foetal age, and no single tooth exhibited the full spectrum of mineralisation equivalent to that of the rat incisor A more complete developmental series must be examined before the full picture emerges but it seems from the teeth illustrated by Fig. 3a–d that, again, the fluoride content of the fully mineralised enamel is lower than that of the partially mineralised tissue.

Independently, R. L. Speirs has now also reported unpublished findings that the fluoride content of developing enamel from pig teeth decreases as the density of the enamel increases

There are several possible explanations for this decrease in fluoride concentration. The first is that fluoride, perhaps acquired by ionic exchange when the enamel is only partially mineralised, might be diluted by the subsequent deposition of relatively fluoride-free enamel. This is

suggested by the rat data in Fig. 2. A similar suggestion was made by Gedalia, Garti and Epstein[21] to explain an observed decrease in the fluoride concentration of developing human teeth between the eighth and ninth months of foetal life. The observed increase in mineral content of the foetal teeth, however, was insufficient to account fully for the decrease in fluoride concentration. In the bovine teeth (Fig. 3) a comparison between the decrease in fluoride concentrations and the concomitant variations in phosphorus content leads also to the conclusion that, here, dilution could not alone explain the fall in fluoride concentration.

Ruzicka and Mrklas (personal communication) suggested that since the fluoride concentration of the skeleton would increase with age, more skeletal fluoride would be released during the normal processes of bone remodelling and the fluoride concentration of the blood would gradually rise with age. This would in turn be reflected in the fluoride concentration of the most recently formed enamel, towards the root apex. Since Ruzicka and Mrklas made this interesting suggestion, it has been shown that the concentration of fluoride in blood does indeed reflect that in the skeleton[22]. Whether this could entirely account for such elevated fluoride concentrations in forming enamel, however, seems doubtful, for the change in the fluoride content of the skeleton over the period of incisor formation would be relatively small, and it would not alone explain the peak concentration of fluoride in the rat incisor just before the final phase of mineralisation; this peak will be mentioned later.
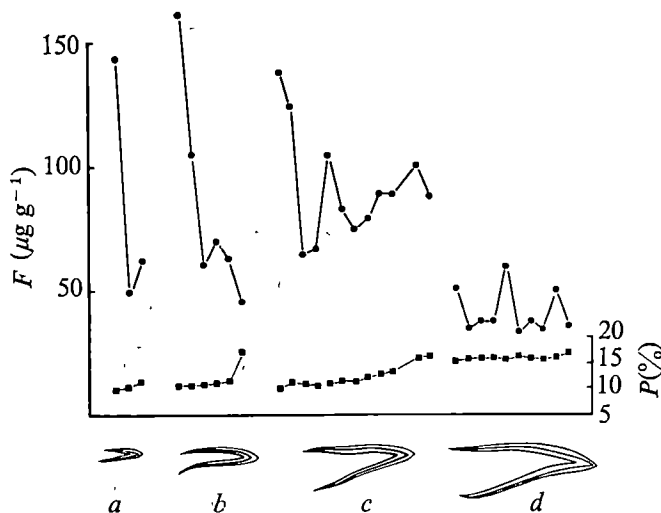
Fig. 3 Distribution of fluoride and phosphorus along the developing enamel of four deciduous bovine incisors taken from foetal calves aged; a, 6.0; b, 6.8; c, 7.8; and d, 8.7 month (dry weight).

The final possibility, and one which seems to have the greatest experimental support at present, is that some of the fluoride acquired during the early stages of enamel development is lost as the tissue mineralises. The ^{18}F studies of Wallace-Durbin[9] showed that the ^{18}F activity of bones and teeth from rats injected with the isotope reached a maximum value and then decreased with time, suggesting that at least some of the fluoride acquired by the mineralising tissue may be lost again. A preliminary attempt by ourselves to relate the fluoride content of the enamel to the volume of the particles dissected from the rat incisor (a procedure intended to eliminate the possibility that the fluoride concentrations fell as a result merely of dilution by the increasing weight of mineral) also supported the view that some of the fluoride acquired during the earlier stages of development is lost as the tissue matures[23].

The recent unpublished findings of Speirs similarly point to fluoride loss.

Although the partially mineralised enamel of the rat incisor had, overall, a higher fluoride concentration than the maturing tissue, there was within this region a fairly consistent pattern of fluoride distribution. A peak of relatively high fluoride concentration usually occurred just before the phase of rapid mineralisation, that is, at or before the boundary between the translucent and opaque enamel, indicated by a V in Fig. 2. The position of this site seems to correspond to a region where ^{32}P-labelled phosphate is preferentially taken up both *in vivo* and *in vitro*[18]. The high ^{32}P activity found in this region seemed to reflect the physicochemical state of the enamel and perhaps fluoride, like phosphate, is incorporated into forming enamel mainly by diffusion into relatively perm-eable tissue. We have no information about the nature of fluoride binding in this highly hydrated region of tissue. The implication in the literature that fluoride is largely bound to the mineral part of enamel is almost certainly justified in the case of mature enamel but is perhaps not without question in this partially mineralised tissue. It seems possible that some of the fluoride might be free, or bound to the organic matrix, as suggested previously by Hammarström[10], and perhaps subsequently lost as part of the matrix is withdrawn from the enamel during mineralisation.

This work was supported by the Medical Research Council.

JOHN A. WEATHERELL
DAN DEUTSCH
COLIN ROBINSON
ALAN S. HALLSWORTH

Department of Oral Biology,
School of Dentistry,
University of Leeds,
Leeds LS2 9NZ, UK

1 Hardwick, J L., *Br. dent. J.*, 119, 304-311 (1965).
2 Rich, C. and Ensinck, J, *Nature*, 191, 184-185 (1961)
3 Jowsey, J., Rigs, B. L., Kelly, P. J., and Hoffman, D. L., *Am. J Med*, 53, 43-49 (1972).
4 Forrest, J. R, *Br. dent. J.*, 119, 316-319 (1965).
5 Hodge, H. C., and Smith, F A., in *Fluorine Chemistry* (edit. by Simons, J H.), 786 (Academic, New York, 1965).
6 Jenkins, G. N., and Speirs, R L., *J. Physiol., Lond.*, 121, 21 (1953)
7 Brudevold, F., Gardener, D. E., and Smith, F A., *J dent Res.*, 35, 420-429 (1956).
8 Gedalia, I , Kfir, Y., and Azaz, B , *J. dent. Res.*, 46, 1465-1468 (1967).
9 Wallace-Durbin, P , *J. dent. Res.*, 33, 789-800 (1954).
10 Hammarstrom, L., *Scand, J. dent. Res.*, 79, 369-376 (1971).
11 Jenkins, G. N., in *Fluoride and Human Health*, 210 (World Health Organization, Geneva, 1970)
12 Hodge, H. C., *Proc. Nutr. Soc.*, 22, 111-117 (1963).
13 Weatherell, J. A., Robinson, C , and Hallsworth, A.S. , in *Tooth Enamel II* (edit. by Fearnhead, R. W., and Stack, M. V.), 31-38 (Wright, Bristol, 1971)
14 Deutsch, D., Weatherell, J A., and Hallsworth, A. S., *J. dent. Res.*, 51, 1278 (1972).
15 Weatherell, J. A., in *Mineral Metabolism in Paediatrics* (edit by Barltrop, D., and Burland, W. L.), 53-70 (Blackwell, Oxford, 1969)
16 Jackson, D., and Weidmann, S M., *Brit. dent. J.*, 107, 303-306 (1959)
17 Gedalia, I., Rosenzweig, K. A , and Sadeh, A., *J. dent. Res.*, 40, 865-869 (1961).
18 Robinson, C., Hiller, C. R., and Weatherell, J. A., *Calc. Tiss. Res.*, 15, 143-152 (1974).
19 Weatherell, J A., Hallsworth, A. S , and Robinson, C , *Archs oral Biol*, 18, 1175-1189 (1973).
20 Chen, P. S., Toribara, T. Y., and Warner, H., *Analyt. Chem.*, 28, 1756-1758 (1956).
21 Gedalia, J , Garti, A., and Lewin-Epstein, J., *Archs oral Biol.*, 12, 1485-1490 (1967).
22 Ericsson, Y., Gydell, K., and Hammarskiold, T., *Int. Res. Commun System*, Category 17, 1, No. 6 (August, 1973).
23 Weatherell, J. A., Deutsch, D., and Hallsworth, A. S., *J. dent. Res.*, 52, 587 (1973).

Growth-dependent changes in terminal heterogeneity involving 3'-adenylate of bacterial 16S ribosomal RNA

THE 3' terminus of the 16S RNA from the small subunit of bacterial ribosomes is directly involved in ribosomal function[1-3]. On the basis of 3'-terminal sequence analysis of bacterial rRNAs, we have proposed that a short sequence of nucleotides in this region has a direct base-pairing role in both the initiation and termination of protein synthesis[4,5].
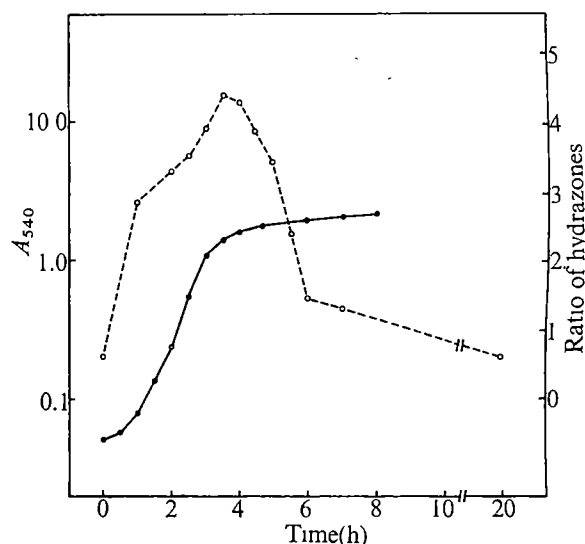
Fig 1. Growth-dependent changes in proportion of *Ps. aeruginosa* 16S rRNA molecules containing a 3'-terminal adenosine residue. *Ps. aeruginosa* was grown in L broth (1% bactotryptone, 0.2% yeast extract, 0.5% glucose, 0.5% NaCl)[8] at 37 °C with vigorous shaking. Samples were taken at intervals, the A_{540} determined, the RNA extracted[9] and the individual rRNA species labelled with ^{3}H-isoniazid[7,9]. The proportion of 16S rRNA molecules terminating in either adenosine or uridine was determined by paper electrophoresis of the labelled nucleoside hydrazones released by pancreatic RNase digestion[4,7]. ●, A_{540}; ○, ratio of c.p.m in adenosine-isoniazid hydrazone to c.p.m. in uridine-isoniazid hydrazone.

This hypothesis is based on two sets of observations. First, the degree of complementarity between the ribosome binding sites of coliphage mRNAs and the pyrimidine-rich 3' sequence of a number of bacterial 16S rRNAs reflects the cistron selectivity of the ribosome[6]. Second, the 3'-terminal trinucleotide of *Escherichia coli* 16S rRNA (UUA$_{OH}$) is the only trinucleotide able to recognise, by standard base pairing, all three nonsense triplets; the strengths of the proposed pairing interactions correlate with the different degrees of suppressibility of the three nonsense codons in suppressing strains of *E. coli*[4]. A similar role has previously been proposed for the highly conserved 3' terminus of eukaryotic 18S rRNA which also terminates in UUA$_{OH}$ (refs 6 and 7).

3'-sequence analysis of rRNA from bacteria other than *E. coli* shows that the 16S rRNA of several species also terminates in UUA$_{OH}$ (ref. 5). 16S rRNA from one member of this group, *Pseudomonas aeruginosa*, however, has a large proportion of molecules which lack the 3'-terminal adenylic acid and two 3' sequences, CUCUCCUUA$_{OH}$ and CUCUCCUU$_{OH}$, are found in approximately equal proportions; no such heterogeneity is found at the 3' termini of 23S rRNA from any bacteria examined (ref. 5 and J. S., unpublished).

As the proportion of 16S rRNA molecules lacking the 3'-terminal adenylic acid varied between different preparations of *Ps. aeruginosa* RNA, we have investigated the influence of bacterial growth conditions on 3'-terminal heterogeneity. In this report we show that the level of 3'-terminal adenylation of *Ps. aeruginosa* 16S rRNA is a function of bacterial growth rate.

Ps. aeruginosa, adapted to L broth[8], was grown at 37 °C with vigorous shaking. Samples were removed at intervals, the cells collected by centrifugation and immediately extracted with phenol-cresol-aminosalicylate[9]. rRNAs (16S and 23S) were separated on sucrose gradients; in all instances the A_{260} ratio of 23S:16S RNA was 2:1. The 3' termini were labelled with ^{3}H-isoniazid and the RNA digested with pancreatic ribonuclease[4,7]. The relative amounts of 3'-terminal adenosine and 3'-terminal uridine in 16S rRNA were determined by paper electrophoresis of the labelled digest[4,7]. The

3'-terminal nucleosides of 23S rRNA were determined by the same method.

Figure 1 shows the proportion of 16S rRNA molecules containing 3'-terminal adenosine, plotted as a function of cell density. The 3'-terminal sequences of the 16S rRNA are initially UUA_{OH} and UU_{OH} in the ratio 0.6:1. By 1 h after inoculation, this ratio had increased to 2.8:1, continued to increase during exponential growth and peaked at approximately 4.5:1 in late logarithmic phase. As the cell growth rate slows the ratio rapidly declines to 1.5:1 in early stationary phase; the rapidity of this change suggests that 3'-adenylate residues are removed from pre-existing 16S rRNA molecules during this period. This ratio falls to 0.6:1 after overnight growth. No significant 3'-terminal heterogeneity was found in 23S rRNA isolated from the same samples; at all stages of the growth curve at least 90% of the 23S molecules terminated in $PyCA_{OH}$.

These results suggest that single adenylate residues may be added post-transcriptionally to the 3' terminus of 16S rRNA *in situ*. Terminal heterogeneity involving adenylic acid has also been observed at the 3' terminus of bacteriophage T7 mRNAs cleaved from a large precursor RNA *in vivo*[10]. The same RNA species, when produced by specific *in vitro* cleavage of the T7 precursor RNA with RNaseIII, contain no detectable 3'-terminal adenosine, suggesting that the 3'-adenylate residues are added post-transcriptionally[11]. Note that these T7 mRNAs also contain a 3'-terminal pyrimidine-rich sequence as is found in 16S rRNA (refs 5, 10 and 11). With T7 mRNAs, the terminal addition of adenylic acid seems to be less specifically regulated than that reported here for 16S rRNA, as between one and three adenylate residues are added to the 3' terminus of the RNA (ref. 10).

In the light of these results, it is of interest that a number of bacteria (including *E. coli* and *Pseudomonas*) and also yeast, contain a ribosome-bound ATP–RNA adenyltransferase[12-17]. *In vitro* this enzyme catalyses the 3'-terminal addition of adenylate residues to RNA and, at least for *Pseudomonas*, demonstrates a preference for the 3' end of rRNA, particularly 16S rRNA, over that of tRNA or homopolyribonucleotides[16]. In yeast, the amount of ribosome-bound enzyme decreases towards late log phase[17].

We have also found substantial 3'-terminal heterogeneity in *Bacillus stearothermophilus* 16S rRNA (ref. 5) and low level heterogeneity at the 3' end of both *B. subtilis* and *Caulobacter crescentus* 16S rRNA. A small proportion ($\sim$12%) of *E. coli* 16S rRNA molecules (which normally terminate in UUA_{OH}) lack a 3'-terminal adenosine when isolated from early stationary phase cells or after a nutritional 'shift-down' (J. S., unpublished). There are previous reports of 3'-terminal heterogeneity in *E. coli* 16S rRNA (ref. 18).

The results presented here may reflect growth-dependent changes in the level or localisation of an enzyme responsible for the addition of 3'-terminal adenylate. Alternatively, changes in the degree of heterogeneity could arise from alterations in accessibility to adenylating enzymes of the 3' terminus of 16S rRNA in different functional states of the ribosome. In view of the suggestion that the 3' trinucleotide may recognise intercistronic nonsense triplets and terminator codons on mRNA (ref. 4), however, a direct regulatory role for the 3'-adenosine in converting UU_{OH} to UUA_{OH} (*E. coli, Ps. aeruginosa*), and CU_{OH} to CUA_{OH} (*B. stearothermophilus, C. crescentus*) should also be considered. Further studies on the functional activity of ribosomes containing 16S rRNA lacking a 3'-adenylate should resolve these questions.

J. Shine
L. Dalgarno

Department of Biochemistry,
School of General Studies,
Australian National University,
Canberra, Australia 2600

1 Senior, B. W., and Holland, I. B., *Proc. natn. Acad. Sci. U.S.A.*, 68, 959–963 (1971).
2 Bowman, C. M., Dahlberg, J. E., Ikemura, T., Konisky, J., and Nomura, M., *Proc. natn. Acad. Sci. U.S.A.*, 68, 964–968 (1971).
3 Sidikaro, J., and Nomura, M., *FEBS Lett.*, 29, 15–19 (1973).
4 Shine, J., and Dalgarno, L., *Proc. natn. Acad Sci. U.S.A.*, 71, 1342–1346. (1974).
5 Shine, J., and Dalgarno, L., *Nature*, 254, 34–38 (1975).
6 Dalgarno, L., and Shine, J., *Nature new Biol.*, 245, 261–262 (1973).
7 Shine, J., and Dalgarno, L., *Biochem J.*, 141, 609–615 (1974).
8 Ramagopal, S., and Subramanian, A. P., *Proc natn. Acad. Sci. U.S.A.*, 71, 2136–2140 (1974).
9 Shine, J., and Dalgarno, L., *J. molec. biol*, 75, 57–72 (1973).
10 Kramer, R. A., Rosenberg, M., and Steitz, J. A., *J. molec. Biol.*, 89, 767–776 (1974).
11 Rosenberg, M., Kramer, R. A., and Stietz, J. A., *J. molec. Biol.*, 89, 777–782 (1974).
12 Gottesman, M. E., Canellakis, Z. N., and Canellakis, E. S., *Biochim. biophys. Acta*, 61, 34–42 (1962).
13 Smith, I., and August, J. T., *J. biol. Chem*, 241, 3525–3532 (1966).
14 Hardy, S. J. S., and Kurland, C. G., *Biochemistry*, 5, 3668–3676 (1966).
15 Payne, K. J., and Boezi, J A., *J. biol. Chem.*, 245, 1378–1387 (1970).
16 Milanino, R., and Chargaff, E., *Proc natn. Acad. Sci. U.S.A.*, 70, 2558–2562 (1973).
17 Twu, J. S., and Bretthauer, R. K., *Biochemistry*, 10, 1576–1582 (1971).
18 Midgley, J. E. M., and McIlreavy, D. J., *Biochim. biophys. Acta*, 142, 345–354 (1967).

Differences in transcription of unique DNA sequences between neuronal and glial cells

OUR previous report[1] provided evidence for the existence of the brain-specific nuclear RNA using the RNA–DNA hybridisation method. As the neuronal and glial cells in the brain possess specific compositions and functions, each of these cells probably contains cell-specific RNA, as suggested by Church and Brown[2]. In fact, Hydén *et al.*[3] found remarkable differences in base composition between the total RNA of neuronal and glial cells. This difference may be accounted for by a greater proportion of cytoplasmic (ribosomal) RNA present in the neurone[4]. This report provides new evidence for the existence of cell-specific RNA in brain, using a unique DNA–³H-RNA hybridisation method.

Fifty minutes after injection of 5,6-³H-uridine (specific activity 43 Ci mmol⁻¹, Radiochemical Centre, Amersham) into the subarachroidal space of a 15–18-d-old rat, the animal was decapitated. Then the brain was removed and homogenised in a solution containing 2.2 M sucrose and 1.5 mM $CaCl_2$, and the homogenate was centrifuged for 60 min at 70,000g. The precipitated nuclei were gently homogenised in 2.0 M sucrose containing 1.5 mM $CaCl_2$ and layered over a discontinuous gradient of 2.2, 2.35, 2.4 and 2.55 M sucrose. The rotor (Hitachi RPS 25-2) was spun for 120 min at 75,000g. The L fraction, recovered at the interface between 2.2 and 2.35 M sucrose, mainly contained neuronal nuclei; and the S fraction, obtained at the bottom, consisted of oligodendroglial nuclei. RNA was extracted from each cell nucleus with a SDS–hot phenol–bentonite procedure[5] and treated with DNase (electrophoretically pure, Worthington, USA) and with Pronase, and finally passed through a Sephadex G50 column. Unlabelled RNA was extracted from the neuronal and glial nuclear fractions by a similar procedure. DNA was isolated from rat brain nuclei by using a modification of Marmur's method[6]. For RNA–DNA hybridisation with a DNA excess, DNA was sonicated to a molecular weight of about 170,000, as estimated by the method of Studier[7], then precipitated with ethanol and further purified by filtration through Sephadex SP50.

To examine transcription in brain cells, two procedures were used: first, RNA–DNA hybridisation, using a membrane filter in the presence of formamide, and immobilisation of DNA on membrane filters (Millipore HAWP)[8]; and second, RNA–DNA hybridisation with excess DNA in solution[9,10]. Figure 1a shows the results obtained with the membrane filter hybridisation technique. When the rapidly labelled RNA of the S fraction was competed with each unlabelled RNA of L and S fractions, almost identical competition curves were obtained with either RNA of the L or S fraction. In the case of labelled RNA of the L frac-
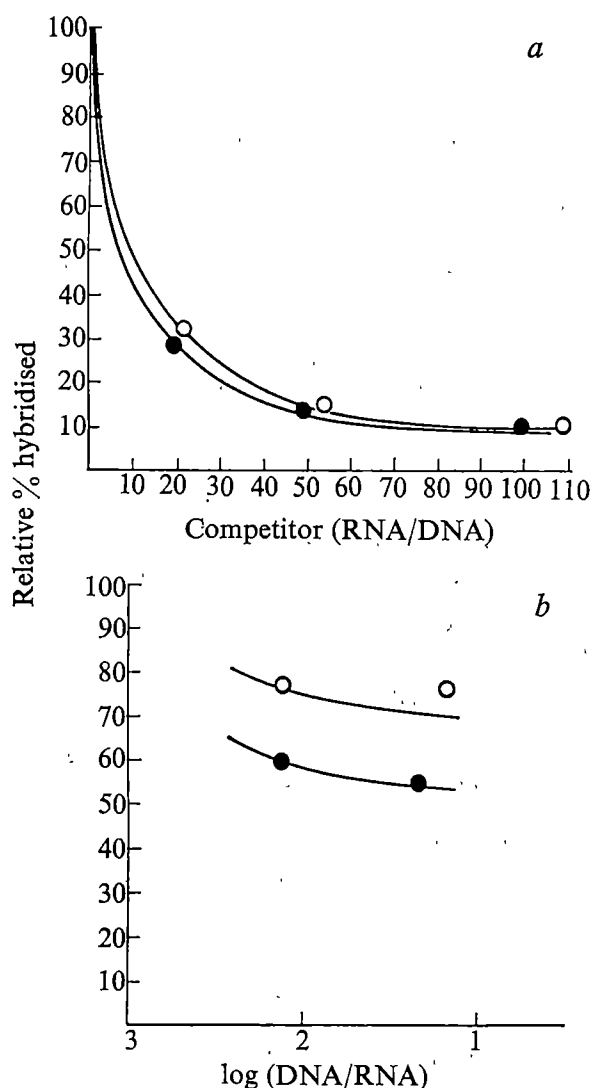
Fig. 1 Competition by unlabelled RNA from L fraction (○) and S fraction (●) in the hybridisation of ³H-labelled RNA from S fraction with brain DNA. *a*, RNA–DNA hybridisation was carried out at 35 °C for 25 h in glass vials in 2 ml 6-SSC–formamide (1:1 v/v). Each incubation medium contained 25 µg ³H-labelled RNA from S fraction (280 c.p.m. µg⁻¹), DNA filter (10 µg) and various amounts of unlabelled competitor RNA. Then the hybrid-containing membrane filters were incubated with RNase and washed on each side. Finally the filters were dried and counted in a scintillation counter. The radioactivity bound to DNA without competitor was 200 c.p.m. and is taken as 100%. *b*, Hybridisation with a DNA excess was carried out by a modified method of Melli *et al.*[9]. DNA fragments in 0.03 M phosphate-buffered saline (PBS) were heated at 100 °C for 10 min. ³H-labelled RNA was added to a single-stranded DNA solution. Reaction mixtures (1 ml) contained 3 mg DNA, 2.94 µg ³H-labelled RNA (1,660 c.p.m.) and unlabelled competitor RNA. The reaction mixture was placed in a water bath at 70 °C and the salt concentration was adjusted to 0.4 M PBS. At C₀*t* 10,000, the incubation reaction was stopped by removing the glass tube and placing it into an ice bath. The hybridisation mixture was diluted with water to a concentration of 0 24 M PBS, and treated with RNase (50 µg ml⁻¹) at 37 °C for 30 min. After RNase treatment the reaction mixture was precipitated with cold 10% TCA. The precipitate was collected on Whatman GF/C glass filter, washed with adequate volumes of 5% TCA and with ethanol, dried and counted in a scintillation counter.

tion, similar results were obtained. As the hybridisation conditions used in these experiments are known to detect differences in the transcription of repeated DNA sequences, the above results may indicate negligible differences in the transcription of the repeated DNA sequences between the L and S fractions.

When the reaction mixture was incubated to C₀*t* 10,000 at 70 °C with a DNA excess, about 40–50% hybridisation was reached in the absence of an unlabelled competitor.

As shown in Fig. 1*b*, when the rapidly labelled RNA of the S fraction was competed with each unlabelled RNA of the L and S fractions, the unlabelled RNA of the S fraction was much more effective in competition than was the L fraction. These results are indicative of significant differences in the transcription of unique DNA sequences between the two cell types. These differences are statistically significant ($P < 0.05$). Therefore, it can be concluded that the neuronal and glial nuclei have the cell-specific RNA species. The proportion of RNA transcribed from repeated DNA sequences seems to be only a minor fraction of the rapidly labelled nuclear RNA and much of this nuclear RNA is transcribed from unique DNA sequences. As some parts of these latter RNA species pass to the cytoplasm as mRNA, cytoplasmic mRNA should be the product transcribed from unique DNA (refs 9 and 11–13). Further, these premessenger nuclear RNAs contain polyadenylate sequences[13,14]. Our present results may indicate the differences in such RNA species between two cell types. On the other hand, no differences are found in RNA transcripts from repeated DNA sequences which seem to be a minor fraction of the heterogeneous nuclear RNA with a small amount of premessenger RNA as described above. The function of such RNA transcribed from repeated DNA is not yet fully elucidated, although the hypothesis of Britten[15] is interesting. As the cytogenetical relationship between two cell types is close[16], it may be difficult to find the differences in such RNA using a membrane filter technique.

Recent reports on the saturation hybridisation experiment have shown that about 8% of mouse unique ³H-DNA is complementary to total brain RNA and that the complexity of the spectrum of RNA from other tissues is about 2%. Church and Brown[2] have suggested that these results reflect the diversity of cell types found in the brain. Our present results may be in agreement with their suggestion. On the basis of the experiment with the total RNA of malignant neural tissues, Grouse *et al.*[17] have reported that neuronal and glial cells exhibit roughly the same extent of transcriptional diversity. Their results may be related to the use of the saturation technique and the total quantities of respective tissues. In the context of our present results, it may be interesting that neurones contain the specific protein like 14, 3, 2 (ref 18) and glia also the specific protein like S100 (ref 18). It will be necessary to isolate and characterise these cell-specific RNAs.

We thank Mr K. Araki and Miss E. Kushiya for assistance.

K. SOGA
Y. TAKAHASHI

*Department of Neuropharmacology,
Brain Research Institute,
Niigata University, Niigata, Japan*

1 Soga, K., and Takahashi, Y., *Abstr Fourth Int. Meeting Int. Soc Neurochem. (Tokyo)*, 287 (1973).
2 Church, R. B., and Brown, I R., in *Nucleic Acid Hybridisation in the Study of Cell Differentiation* (edit. by Ursprung, H), 11–24 (Springer, Berlin 1972)
3 Hydén, H., and Egyhazi, E , *Proc natn. Acad. Sci. U.S.A.*, 49, 618–624 (1963).
4 Rose, S P. R , in *Applied Neurochemistry* (edit. by Davison, A. N , and Dobbing, J.), 348 (Blackwell, London, 1968).
5 Takahashi, Y., Araki, K., and Suzuki, Y., *Brain Res.*, 32, 179–188 (1971).
6 Marmur, J., *J molec Biol* , 3, 208–218 (1961).
7 Studier, F., *J. molec. Biol* , 11, 373–380 (1965)
8 Gillespie, D., and Spiegelman, S ,*J molec Biol.*, 12, 829–842 (1965).
9 Melli, M., Whitfield, C., Roa, K V., Richardson, M., and Bishop, J. O., *Nature new Biol* , 231, 8–12 (1971).
10 Church, R. B., in *Molecular Techniques and Approaches in Developmental Biology* (edit. by Chrispeels, M J), 223–301 (Wiley, New York, 1973).
11 Greenberg, J R., and Perry, R. P., *J. Cell Biol.*, 50, 774–786 (1971).
12 Smith, M. J., Hough, B. R , Chamberlin, M. E , and Davidson, E. H., *J. molec. Biol.*, 85, 103–126 (1974).
13 Klein, W. H., Murphy, W , Attardi, G., Britten, R. J., and Davidson, E. H., *Proc natn. Acad. Sci U.S A.*, 71, 1785–1789 (1974).
14 Bantle, J , and Hahn, W. E., *J. Cell Biol* , 63, 14a (1974).
15 Britten, R. J , and Davidson, E H., *Science*, 165, 349–357 (1969).
16 Fujita, S , in *Evolution of the Forebrain* (edit by Hassler, R., and Stephan, H.), 180–196 (Thieme, Stuttgart, 1966)
17 Grouse, L., Omenn, G. A., and McCarthy, B. J., *J. Neurochem.*, 20, 1063–1073 (1973).
18 Moore, B W., in *Proteins of the Nervous System* (edit. by Schneider, D. J.), 1–12 (Raven, New York, 1973).

Excision of thymine dimers from specifically incised DNA by extracts of xeroderma pigmentosum cells

SINCE the initial observations on defective DNA repair in fibroblasts cultured from patients with xeroderma pigmentosum (XP)[1,2], a large number of cases have been collected and studied in laboratories around the world The results of these studies indicate a surprising degree of complexity in the DNA repair defect(s) in this disease. At an early stage it became apparent that not all cell strains are totally defective in the excision repair of ultraviolet radiation damage in vivo, there being varying degrees of "leakiness"[3-5]. Cell fusion studies have since indicated that a number of distinct genetic complementation groups exist, suggesting a multigenic basis for the disease[6-9]. In addition, defects in photoreactivation[10] and in post-replication repair[11] of ultraviolet radiation damage in some XP strains have been reported.

If we confine our attention to those strains of XP cells that exhibit defects in excision-repair (ignoring for this study the variants which have normal excision repair[12,13] but defective post-replication repair[11]) then five distinct complementation groups, designated A,B,C,D,E, have been identified[6,7]. Only one patient in group B is currently known.

At present, little is known about the biochemical basis of the repair defectiveness of XP cells in culture, or of the various complementation groups. In all cases specifically examined, indirect studies suggest the presence of an endonuclease or endonuclease-related defect. The evidence on which this suggestion has been made includes the ability of XP cells to repair DNA strand breaks from ionising radiation[14,15] and alkylating agents[16,17], the failure of XP cells to repair damage to DNA bases that require endonucleolytic incision[17-19] and the failure of XP cells to excise dimers[2,20] or remove ultraviolet endonuclease-sensitive sites[21] from their DNA Direct demonstration of a defect in the incision step of repair by measurement of single strand breaks in DNA has not been achieved[22,23].

Since many of these studies were carried out in different laboratories, before complementation analysis began, it is not possible to state whether an apparent incision defect is characteristic of all complementation groups, but no exceptions have been reported except for the XP variant. Current understanding of the molecular mechanism of excision repair based on prokaryote models, calls for an endonucleolytic incision of DNA, excision of the damaged segment and repair synthesis. One might therefore expect that some cases of XP would show defects in the latter two steps. Nonetheless, the finding of a DNA incision defect in more than one complementation group is not inconsistent with the existence of multiple alleles, if the human cell ultraviolet endonuclease has multiple subunits and/or if other gene products are required for the endonuclease to function in human cells.

An alternative hypothesis first suggested by Haynes[24] is that the repair enzymes may function as a complex in a coordinated way, such that a defect in any one step would block the entire repair process. If this hypothesis were correct, then cases of XP defective in an excision nuclease or a DNA polymerase activity would still show a defect in the incision of ultraviolet-irradiated DNA and in vitro none of the enzymatic steps associated with excision repair should be demonstrable On the basis of this kind of model, it has been suggested that all the data currently available on XP cells is consistent with either a defective endonuclease or exonuclease[25].

To obtain direct evidence for the validity of the enzyme complex hypothesis and further information on the repair

Table 1 Characteristics of XP cell lines used for dimer excision ability

Cell line	Neurological symptoms	Level of unscheduled synthesis*	Complementation group
XP 12 (SV40)	yes	0	A†
XP 25 RO	yes	0	A†
XP1 PW	no	4-7%	C‡
XP2 NE	yes	33-51%	D‡
XP 21 SF	no	60-65%	E‡

*Measured by autoradiography of fibroblasts labelled for 3 h with ³H-thymidine (10 μCi ml⁻¹ 15.8 Ci mmol⁻¹) after irradiation with 50–100 erg mm⁻².
†Assigned by complementation experiments[6,8].
‡Assigned by comparison of clinical symptons of neurological involvement and levels of unscheduled synthesis with the groups described elsewhere[6,7].

defects in XP cells, we have developed a cell-free system that excises pyrimidine dimers in vitro[26]. In our initial investigations[26,27] we reported the presence of an exonuclease activity in extracts of normal human cells in culture, which excises thymine dimers from ultraviolet-irradiated DNA previously incised with a dimer-specific endonuclease purified from bacteriophage T4-infected E. coli[28]. This activity excises thymine dimers in the 5'→3' direction, is stable to freezing, has a requirement for Mg^{2+} or Mn^{2+} and is sensitive to inhibition by SH-blocking agents. A similar activity has recently been purified from human placenta[29]. We have now examined extracts of XP cells for dimer excising activity on previously incised DNA from four of the complementation groups discussed above. Fig. 1 shows the results obtained with extracts of a human cell line (HeLa) and an XP cell line (XP 12 [SV40] RO). Table 2 shows the excision of thymine dimers by extracts of a normal and four XP fibroblast strains. The results of a number of such experiments indicate no significant differences in the specific activity of excision nuclease in any of the XP classes compared with normal cells.

These results lead us to conclude, first, that the hypothesis of a coordinated enzyme complex for performing excision repair is not applicable to excision of pyrimidine dimers in vitro: extracts of all XP classes can excise pyrimidine dimers from an exogenous DNA when the first step of repair is completed by the T4 phage coded endonuclease. Second, there seem to be no examples of XP cells defective in the excision step of excision repair in vitro. This lends further support to the observation that the biochemical defects in excision repair in this disease may be related to events pertaining to endonucleolytic incision of damaged DNA.

We offer these conclusions with the following reserva-

Fig. 1 Details of the experimental procedures are described in the legend to Table 2. Each incubation contained crude protein in the amount indicated. ○, ×P 12 (SV) RO; ●, HeLa.
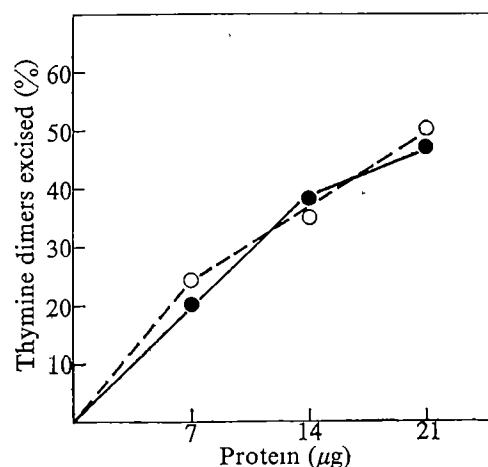
Table 2 Thymine dimer excision activity in normal and XP cell extracts

Source extract	Complementa-tion group	Thymine dimers excised in 30 min (%)	Total acid-soluble nucleotides (%)
197 P (normal)	—	22	5.4
XP 25 RO	A	20	3.4
XP 1 PW	C	19	4.8
XP 2 NE	D	32	2.8
XP 21 SF	E	25	5.2

XP cells were obtained by skin punch biopsy and the normal fibroblast strain (197P) was obtained from foreskin. The cells were grown in minimal Eagles medium with 15% foetal calf serum (Gibco, Grand Island, NY) and 100 U ml^{-1} of penicillin and streptomycin at 37 °C in a 5% CO_2 atmosphere. Cells were collected with trypsin-EDTA and washed three times with buffered saline. Cell cultures were checked for mycoplasma by the method of Todaro *et al.*[30] and showed no contamination. Cell-free extracts (microscopic examination revealed complete disruption of cells and nuclei) were made in 0.15 M NaCl, 0 01 M Tris-HCl buffer *p*H 8.0 and 0.005 M β-mercaptoethanol with| 30 strokes of a Dounce homogeniser using a tight-fitting pestle. Incubation mixtures (0.1 ml) contained 22.5 nmol of ^3H-*E. coli* DNA previously ultraviolet-irradiated at a fluence of 334 J m^{-2} and incised with T4 ultraviolet endonuclease[28]. This DNA was shown to have endonucleolytic incisions on the 5' side of 80–95% of the thymine dimer sites. Also present in the mixture were $MgCl_2$, 0.1 M; Tris-HCl buffer, *p*H 8.0, 0.01 M and 140 μg ml^{-1} protein from the crude extract. Incubation was at 37 °C for 30 min. Reactions were terminated by the addition of 0.1 ml 10% cold trichloroacetic acid. The acid-insoluble fractions were hydrolysed and thymine dimer contents determined as described previously[31].

tions in mind. First, these studies have used cell-free preparations and we have no direct evidence that the enzyme activity or activities measured, function in dimer excision *in vivo*. Second, the substrate used consists of ultraviolet-irradiated *E. coli* DNA incised with an endonuclease purified from phage-infected cells. It is possible that the human UV endonuclease acts in a manner which precludes the action of the nuclease activity we have measured and creates substrate sites for a different enzyme. It is also possible that the excision step involves an additional factor(s) in order to accommodate the complexity of the chromatin substrate in living cells.

K. Cook
E. C. Friedberg
J. E. Cleaver*

Laboratory of Experimental Oncology,
Department of Pathology,
Stanford University School of Medicine
Laboratory of Radiobiology,
University of California School of Medicine,
San Francisco, California 94305

1 Cleaver, J E., *Nature*, 218, 652 (1968).
2 Setlow, R. B., Regan, J. D , German, J., and Carrier, W. L , *Proc. natn. Acad Sci. U.S.A.*, 64, 1035 (1969).
3 Bootsma, D., Mulder, M. P., Pot, F , and Cohen, J. A., *Mutat. Res.*, 9, 507 (1970).
4 Cleaver, J. E., *J. Invest. Dermatol.*, 54, 181 (1970).
5 Cleaver, J. E , *Int. J. radiat. Biol.*, 18, 557 (1970).
6 Cleaver, J. E., and Bootsma, D , *Adv. Genet.*, 9 (in the press).
7 Kraemer, K. H., *et al*, *Proc. natn. Acad. Sci. U.S.A.*, 72, 59 (1975)
8 de Weerd-Kastelein, E. A., Keijzer, W., and Bootsma, D., *Nature new Biol*, 238, 80 (1972).
9 de Weerd-Kastelein, E. A., Keijzer, W., and Bootsma, D., *Mutat. Res.*, 22, 87 (1974).
10 Sutherland, B. M., Rice, M., and Wagner, E. K., *Proc. natn. Acad Sci. U.S A.*, 72, 103 (1975)
11 Lehman, A. R., *et al.*, *Proc. natn Acad. Sci. U.S.A.*, 72, 219 (1975).
12 Burk, P. G., Lutzner, M. A., Clarke, D D., and Robbins, J. H., *J. Lab. Clin. Med.*, 77, 759 (1971).
13 Cleaver, J. E., *J Invest. Dermatol.*, 58, 124 (1972)
14 Cleaver, J. E., *Proc. natn. Acad. Sci. U.S.A.*, 63, 428 (1969).
15 Kleijer, W. J , Lohman, P. H. M., Mulder, M. P , and Bootsma, D., *Mutat. Res.*, 9, 517 (1970).
16 Cleaver, J. E., *Mutat. Res.*, 12, 453 (1971).
17 Cleaver, J. E., *Cancer Res*, 33, 362 (1973).
18 Setlow, R. B , and Regan, J. D., *Biochem. biophys. Res Commun.*, 46, 1019 (1972).
19 Stich, H F., San, R H. C., Miller, J. A , and Miller, E. C., *Nature new Biol*, 238, 9 (1972).
20 Cleaver, J. E., and Trosko, J. E., *Photochem Photobiol.*, 11, 547 (1970).
21 Paterson, M. C., Lohman, P H. M , and Sluyter, M. L., *Mutat. Res*, 19, 245 (1973).
22 Cleaver, J. E., *Radiat. Res.*, 57, 207 (1974)
23 Kleijer, W. J., Hoeksema, J. L., Sluyter, M. L , and Bootsma, D., *Mutat. Res.*, 17, 385 (1973).
24 Haynes, R. H , *Radiat. Res. Suppl.*, 6, 232 (1966).
25 Cleaver, J. E., in *Advances in Radiation Biology* (edit. by Lett, J. T., Adler, H , Zelle, M.), 4, 1 (Academic, New York, 1974).
26 Friedberg, E. C., Duncan, J., and Cleaver, J. E., *Radiat. Res.*, 59, 98 (Abstr.) (1974).
27 Duncan, J., *et al.*, in *Molecular Mechanisms for the Repair of DNA* (edit. by Setlow, R B., and Hanawalt, P. C.) (Plenum, in the press).
28 Friedberg, E. C , and King, J J., *J. Bact.*, 106, 500 (1971).
29 Doniger, J., and Grossman, L., *Biophys. J.*, 15, 297a (Abstr.) (1975).
30 Todaro, G. J., Aaronson, S. A., and Rands, Elaine, *Expl Cell Res*, 65, 256 (1971).
31 Goldmann, K., and Friedberg, E. C , *Analyt. Biochem*, 53, 124 (1973).

Regeneration of the photoprotein aequorin

THE photoprotein aequorin (molecular weight about 30,000) isolated from the bioluminescent jellyfish *Aequorea aequorea*[1-3] emits blue light (λ_{max} 470 nm)[4] by an intramolecular reaction in aqueous solution when Ca^{2+} is added, in the presence or absence of molecular oxygen[1]. This reaction has been postulated[5] to follow the scheme in (Fig. 1), in which an essential component H_2O_2 of aequorin is bound to the protein part in a form such as a peroxyacid or an α-hydroxyhydroperoxide which readily releases H_2O_2. Hypothesised roles for peroxide in aequorin (see recent review[6]) are consistent with this scheme. YC is a yellow chromophore of unknown structure, responsible for the colour of aequorin from which it has been isolated[5], and found to be different from other components in Fig. 1.

The luminescence reaction requires the binding of three Ca^{2+} to one molecule of aequorin[4]. The product is a blue fluorescent protein (BFP) that exhibits a fluorescence spectrum exactly corresponding to the blue luminescence of the Ca^{2+} triggered reaction[4]. In BFP, the light-emitting moiety (Fig. 1, compound I), which is not fluorescent itself in aqueous solution, binds to the protein part (dissociation constant 5×10^{-6} M)[7] yielding the fluorescent BFP (ref. 8). We have now found that the protein moiety of spent aequorin reacts with compound II in the presence of molecular oxygen, leading to regeneration of aequorin (Fig. 1). Compound II, initially found in aequorin in its enolised structure[5], is evidently the same as natural luciferin of *Renilla*[9]. We have named this compound coelenterazine[9] in view of its widespread occurrence in luminescence systems of coelenterates[6,9,10].

A sample of electrophoretically pure aequorin[3,4] was luminesced by addition of Ca^{2+}, and the resulting BFP was passed through a column of Sephadex G25 (Pharmacia) prepared with 0.01 M Tris-HCl buffer (*p*H 7.5) containing 0.01 M EDTA, to separate the protein moiety and compound I. The former eluted in the void volume, whereas the latter was adsorbed on the column. Treatment of this protein with a large excess of synthetic compound II (ref. 11), in the presence of molecular oxygen plus 2 mM 2-mercaptoethanol and 5 mM EDTA, at 5 °C, gave rise to Ca^{2+}-triggerable photoprotein. The yield of photoprotein was 50% after 25–30 min, and nearly 90% within 3 h, with reference to the amount of protein molecules. After 20 h the reaction mixture was passed through a column of Sephadex G25 (prepared as above) to separate photoprotein from reagents. The photoprotein thus obtained was identical to the original aequorin as evinced by absorption spectrum (λ_{max} 281 and 460 nm, $A_{460}/A_{281} = 0.030$), luminescence spectrum (λ_{max} 470 nm), luminescence activity (1.55×10^{15} photons ml^{-1} at 25 °C when A_{280} is 1.0 cm^{-1}), kinetics of luminescence in the presence and absence of air (Fig. 2), and chromatographic behaviour on DEAE-cellulose and Sephadex G100. Regeneration is thus confirmed; it was not influenced by various concentrations of EDTA or by the addition of H_2O_2, but it did not take place in an evacuated reaction vessel without oxygen.

When compound II was added to a solution of separated
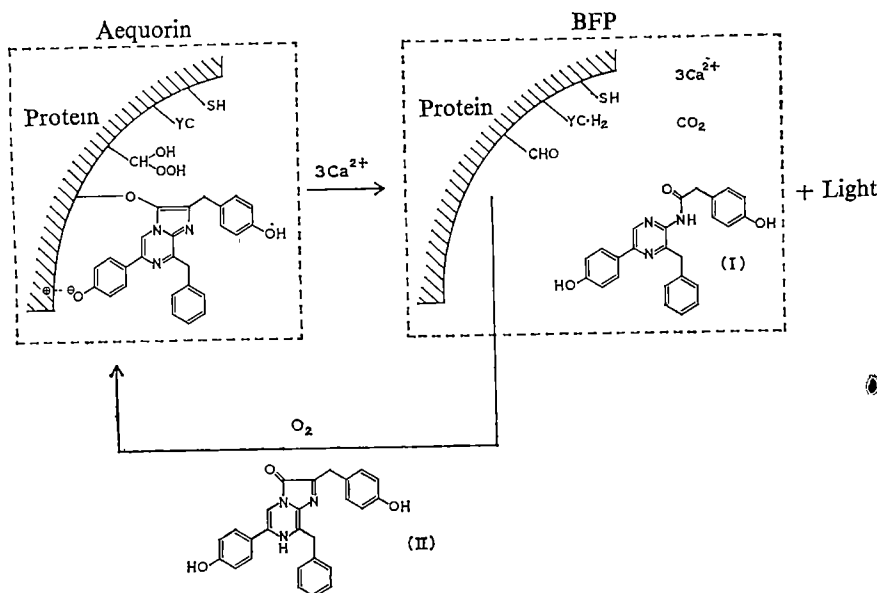
Fig. 1 Postulated mechanism of luminescence[5] and regeneration of aequorin. Bound H_2O_2 in aequorin is tentatively shown as α-hydroxyhydroperoxide. After the luminescence reaction, YC in aequorin is reduced to a colourless form $YC \cdot H_2$, but the sulphydryl (SH) group(s) essential for light emission is ultimately unchanged. CO_2 in BFP may be bound to the protein.

protein moiety together with Ca^{2+}, or directly into spent aequorin, that is, into the solution of BFP containing Ca^{2+}, aequorin was regenerated and luminesced simultaneously as Ca^{2+} was already present. The result was thus a weak, continuous luminescence (Fig. 3). The rate of slow light emission in such circumstances obviously parallels the rate of regeneration of aequorin, in as much as the usual Ca^{2+}-triggered luminescence of aequorin is very fast (see Fig. 2). In the presence of 2-mercaptoethanol plus an excess of II, the weak luminescence continued for many hours, resulting in a total photon emission at least five times that of the fast luminescence of the aequorin originally used. In the absence of 2-mercaptoethanol, however, luminescence gradually died away, resulting in a total photon emission only 10-15% that which occurs in the presence of 2-mercaptoethanol. Thus, this reagent seems to protect the functional sulphydryl group(s) during regeneration.

According to the above, the protein moiety of spent aequorin

Fig. 2 Ca^{2+}-triggered luminescence of native aequorin (○) and regenerated aequorin (●). Curves for 10^{-2} (a), 10^{-4} (b) and 10^{-6} M Ca^{2+} (c) are offset at 1, 2, and 3 s, respectively, for 0 time. Luminescence was initiated by quick addition of 3 ml 10 mM Tris-HCl buffer (pH 7.2) containing calcium acetate to aequorin solutions consisting of 3 μl aequorin stock solution containing 0.2 mM EDTA (A_{280} 1.44 cm^{-1} for both native and regenerated aequorin) plus 0.3 ml water. To avoid contamination of the more dilute (10^{-4}, 10^{-6} M) Ca^{2+} solutions, plastic rather than glass containers and pipettes were used throughout, except for a Hamilton syringe to measure the aequorin stock solution. The absence of any effect due to the presence of oxygen is shown by data (▲) on luminescence of regenerated aequorin with 10^{-2} M Ca^{2+} in a vessel thoroughly evacuated by a vacuum pump. Temperature: 24 °C.
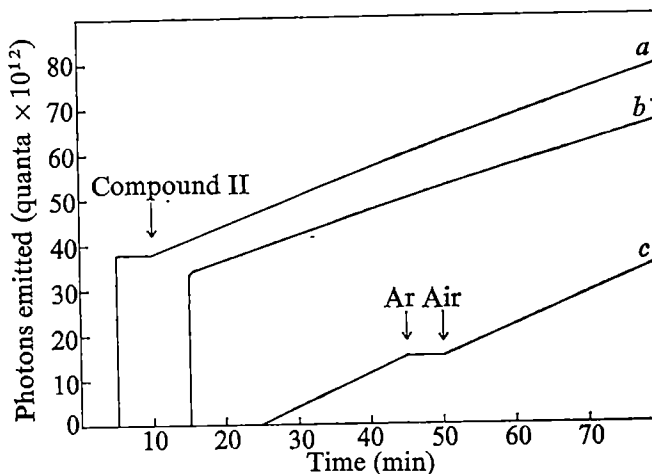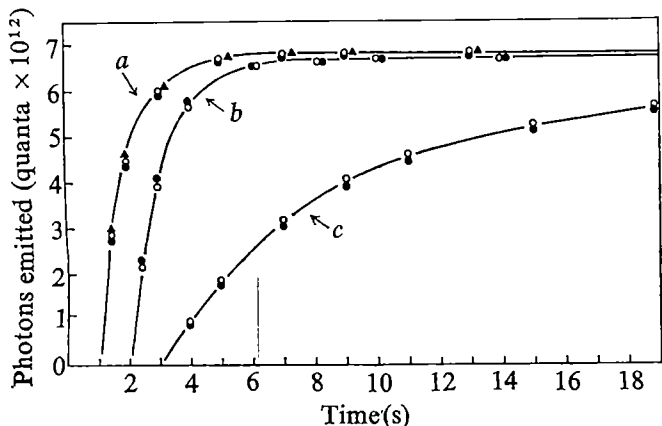
Fig. 3 Ca^{2+}-triggered luminescence of aequorin and regenerated aequorin: a, 4 ml 10 mM Ca acetate was added to 8.7 μg aequorin in 1 ml buffer at 5 min, followed by addition of 6 μg II in 20 μl methanol at 10 min; b, 8.7 μg protein moiety of BFP and 6 μg II were added to 1 ml buffer; the mixture was kept at 5° C for 3 h, then placed in the light-measuring apparatus at 0 min, and 4 ml 10 mM Ca acetate was added to this mixture at 15 min; c, 4 ml 10 mM Ca acetate containing 6 μg II was added to 1 ml buffer containing 8.7 μg protein moiety of BFP at 25 min; bubbling the mixture with Ar gas started at 45 min; Ar was switched to air at 50 min, then bubbling was stopped after 15 s. The buffer (pH 7.5) in all cases consisted of 10 mM Tris-HCl, 5 mM EDTA and 2 mM 2-mercaptoethanol. The amount of aequorin was estimated from A_{280} ($E1\%$-1cm = 27.0) (ref. 2). The amount of protein moiety of BFP was calculated assuming that aequorin yielded the same amount of the protein moiety. All luminescence measurements were carried out at 20 °C. The quick flash at 5 min is caused by native aequorin; all other light emissions are caused by regenerated aequorin.

can be regarded as an enzyme catalysing a luminescent oxidation of coelenterazine (II). In this view, aequorin itself must be considered to represent an intermediate, as hypothetically suggested by several investigators[6]. If so, by its known properties it is an indissociable intermediate[1,2] that is extraordinarily stable (in the absence of Ca^{2+}).

The extent to which regeneration takes place in the organism is uncertain, but the presence of coelenterazine enol-sulphate in the photogenic tissues of Aequorea in an amount equivalent to as much as 5% of the aequorin molecules[9] suggests that the photoprotein recycles in vivo as well as in vitro. Terminal steps in the generation of aequorin in vivo possibly follow the same pathway as regeneration.

The above results are interesting also in regard to the use of

aequorin luminescence as a fast, sensitive test for presence of Ca^{2+} in biological systems[12-19]. Inclusion of coelenterazine in the test system should prolong considerably the luminescence life of aequorin.

A recent paper[20] contains statements which amount to the suggestion that YC of the present paper is in effect *Renilla* luciferin. We have shown, however, that *Renilla* luciferin is actually identical to coelenterazine[9]. It should be emphasised that the absorption spectrum of YC (ref. 5) is significantly different from that of coelenterazine except for the one peak in the visible region. Moreover, recent mass spectrometric data (O.S. and F.H.J., unpublished) have demonstrated that YC and coelenterazine are in fact different compounds.

We thank Drs T. Goto and S. Inoue for a sample of synthetic coelenterazine, and the US National Science Foundation and Office of Naval Research for financial support.

OSAMU SHIMOMURA
FRANK H. JOHNSON

*Biology Department, Princeton University,
Princeton, New Jersey 08540*

1 Shimomura, O., Johnson, F. H., and Saiga, Y., *J. cell. comp. Physiol.*, 59, 223–240 (1962)
2 Shimomura, O., and Johnson, F. H., *Biochemistry*, 8, 3991–3997 (1969).
3 Kohama, Y, Shimomura, O., and Johnson, F. H., *Biochemistry*, 10, 4149–4152 (1971).
4 Shimomura, O., and Johnson, F H., *Nature*, 227, 1356–1357 (1970).
5 Shimomura, O , Johnson, F. H., and Morise, H., *Biochemistry*, 13, 3278–3286 (1974).
6 Cormier, M. J., Hori, K., and Anderson, J. M., *Biochem. biophys. Acta*, 346, 137–164 (1974).
7 Morise, H., Shimomura, O., and Johnson, F. H., *Biochemistry*, 13, 2656–2662 (1974)
8 Shimomura, O., and Johnson, F. H., *Tetrahedron Lett.*, 2963–2966 (1973).
9 Shimomura, O , and Johnson, F H., *Proc natn. Acad Sci U.S.A* , 72, 1546–1549 (1975)
10 Cormier, M. J., et al., *J. cell. Physiol.*, 81, 291–298 (1973).
11 Inoue, S., et al., *Chem. Lett.*, 141–144 (1975).
12 Ashley, C. C., and Ridgway, E. B., *Nature*, 219, 1168–1169 (1968).
13 Azzi, A , and Chance, B., *Biochim. biophys. Acta*, 189, 141–151 (1969).
14 Baker, P. F., Hodgkin, A. L., Ridgway, E. B., *J. Physiol., Lond.*, 218, 709–755 (1971).
15 Johnson, F H , and Shimomura, O., *Nature new Biol.* 237, 287–288 (1972).
16 Llinas, R., Blinks, J. R., and Nicholson, C., *Science*, 176, 1127–1129 (1972)
17 Kaminer, B., and Kimura, J., *Science*, 176, 406–407 (1972).
18 Stinnakre, J., and Tauc, L., *Nature new Biol.*, 242, 113–115 (1973).
19 Chang, J. J., Gelperin, A , and Johnson, F. H., *Brain Res.*, 77, 431–442 (1974)
20 Hori, K., Anderson, J. M., Ward, W. W., and Cormier, M. J., *Biochemistry*, 14, 2371–2376 (1975).

Erratum

In the article "Computer simulation of protein folding" by M. Levitt and A. Warshel (*Nature*, **253**, 694; 1975) the wrong version of Fig. 4 was printed. The correct version is reprinted below.
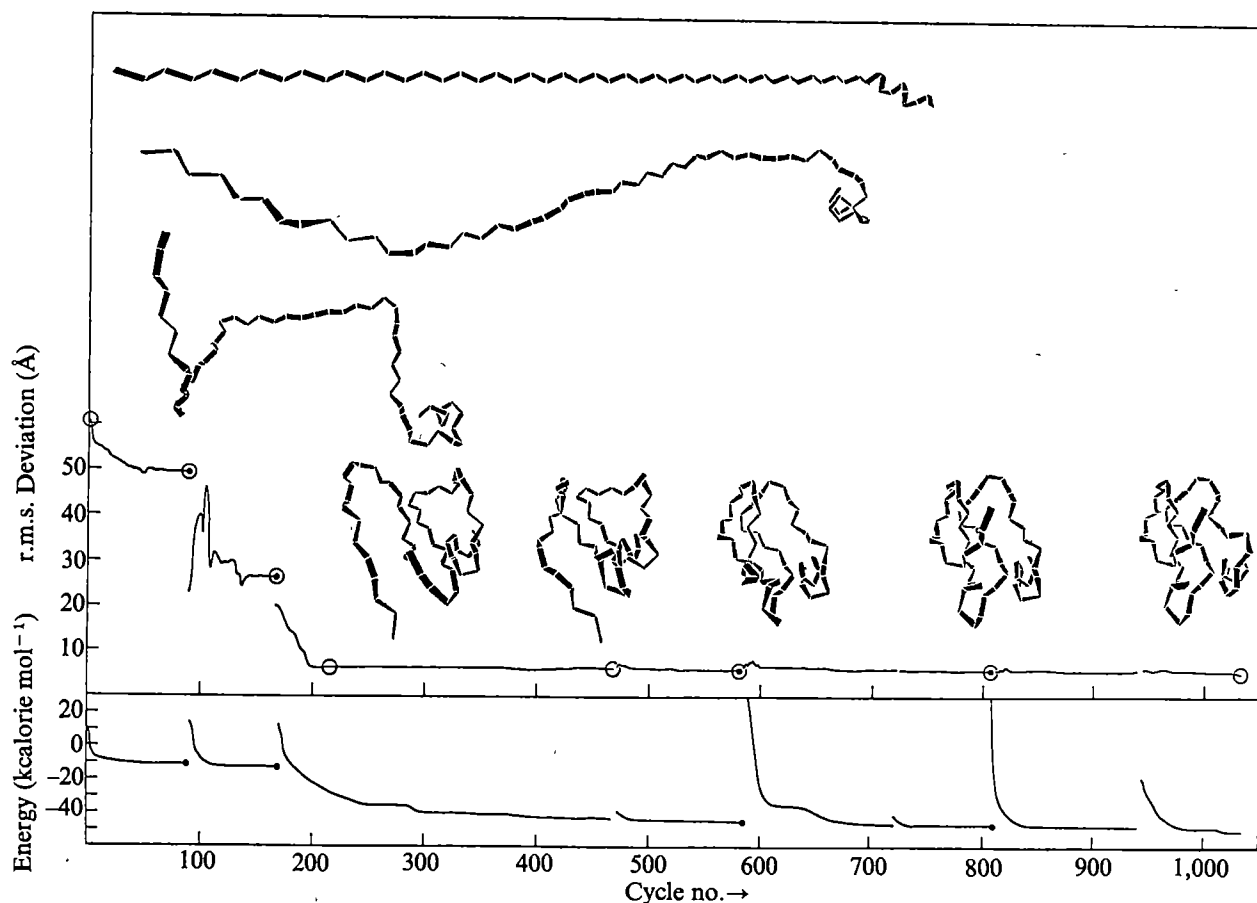
Fig. 4 Simulation of PTI folding from an extended starting conformation with the terminal helix ($\alpha = 180°$ for all except 48 to 58 where $\alpha = 45°$). No knowledge whatsoever about native PTI is used during this simulation (apart from setting the terminal helix). The conformation was thermalised at the end of each minimisation except near cycles 490 and 730 when the energy rises slightly because the minimisation was restarted after rounding the torsion angles to one degree. In the first two thermalisations, each normal mode was perturbed in the plus direction to raise the associated energy to $R(n)kT/2$ with $T = 1,000$ K. In the other three thermalisations, the perturbations were randomly in the plus and minus directions but always such as to raise the energy by $kT/2$ with $T = 300$ K. (Because the random numbers are distributed uniformly rather than exponentially, these temperatures do not correspond to the macroscopic temperature.) The 8 ribbon diagrams, which show the C^α chain path, refer from left to right to the 8 conformations at the circled points on the r.m.s. deviation curve, respectively. The last five conformations have progressively lower energies and are each a little closer to the native structure ($E = -43.3$, -45.7, -46.0, -46.9 and -48.9 kcalorie mol^{-1}, respectively; r.m.s. deviation $= 6.08$, 5.7, 5.6, 5.4 and 5.3 Å, respectively). The solid dots at the end of a minimisation indicate that a perfect minimum was reached (r.m.s. gradient less than 10^{-6} kcalorie $mol^{-1} rad^{-1}$). One cycle takes about 0.6 s on an IBM 370/165 computer.

matters arising

PCB concentrations in North Atlantic surface water

CONCENTRATIONS of polychlorobiphenyls (PCBs) have supposedly[1] declined 40-fold over wide regions of the North Atlantic from levels of about 30 ng l⁻¹ in 1971–72, to 0.8 ng l⁻¹ in 1973–74. This decline has been attributed[1] to the restriction of sales of PCB for non-captive uses during the period 1970–73, both in Europe and in North America. Much as we would like to believe these conclusions, they seem unfounded to us, and they seem to ignore the dynamics of the PCB–seawater system.

From estimates of PCB concentrations in 0.3 µm filtered seawater from 41 North Atlantic stations, Harvey et al. estimated a standing stock of dissolved PCB in the upper 200 m of the North Atlantic of 2×10^4 tonnes (t) in 1972 (ref. 2) in addition to whatever quantity was bound up with <0.3 µm of particulate material; they suggested that the latter could increase the amount of PCB in the 1972 standing stock by 10%.

Using the reported 40-fold decline in the PCB concentration, and a model based on a simple exponential loss-rate we have derived a mean residence time, r, for PCB in seawater in the North Atlantic. Our model simulates the loss process on a daily basis by reducing the standing stock, S, by S/r. A range of values of r was tested to discover the value which reduced S ($= 2 \times 10^4$) by 97.5% ($= 40$-fold) at the end of 365 d. The model indicated a mean residence-time of just under 100 d. In order for such a system to sustain the reported standing stock of 2×10^4 t, this value of r requires an annual input of 7.3×10^4 t (representing the loss from the stock over 365 d).

That seems absurd in relation to what is known about the total production of PCB during the period 1960–71 (Table 1) because it suggests that a standing stock

results are not correct, so that the 1971–72 data (24–41 µg l⁻¹) are too high or the 1973–74 data (0.8–2.0 µg l⁻¹) are too low; or, second, that the extrapolation from individually correct data to a standing stock for the whole North Atlantic is at fault.

It seems relevant to note that three different analytical methods were used to obtain the two sets of data, that no evidence is presented on the repeatability of individual data and that, at least for the 1971–72 data, 36 of the 52 samples were collected with buckets at the water surface and so must have included an unknown and variable quantity of material from the surface microlayer which is known to be highly enriched with hydrocarbons even in the open ocean[3].

Further, if the results from the 1971–72 analyses proved to be too high to be representative of water between the surface and a depth of 200 m over the North Atlantic as a whole, it would also resolve the paradox that has been evident for some years between those data and the relatively low values (about 1.0 ng l⁻¹) reported from coastal waters of the generally rather contaminated California Current[4] and those (<10.0 ng l⁻¹) from the Irish Sea[5], a region likely to be relatively highly contaminated.

The extrapolation from the two sets of data to two levels of standing stock for the whole North Atlantic (which is based simply on an estimate of the volume of the 0–200 m layer of that ocean) also seems to be not very well founded. The data are log-normally distributed, and so extrapolation would have been more appropriate from the geometric rather than the arithmetic mean; such a statistical distribution is likely to be a result of the very high degree of variability within regions (Harvey[2] reports a range of 1–150 µg l⁻¹ over a distance of only 80 km in the open Atlantic) or to the fact that both the

Quinn et al.[3] are widely separated: in 1971 they came from a line between Newfoundland and Iceland, but in 1973 they came from the Sargasso Sea. We therefore suggest that the data and interpretations presented by Harvey et al.[1] are insufficient evidence on which to base the deduction that recent partial controls on uses of PCBs have already produced a dramatic decrease in the contamination of the upper layers of the open Atlantic Ocean.

ALAN R. LONGHURST
PHILIP J. RADFORD

*Institute for Marine Environmental
Research,
Plymouth PL1 3DH, UK*

1 Harvey, G. R., Steinhauer, W. G., and Miklas, H. P., *Nature*, **252**, 387 (1974).
2 Harvey, G R., Steinhauer, W. G., and Teal, J. M., *Science*, **180**, 643 (1973).
3 Bidleman, T. F., and Olney, C. E , *Science*, **183**, 516 (1974).
4 Goldberg, E., *NSF IDOE Baseline Conference*, 11 (National Science Foundation, Washington, DC, 1972).
5 *The Seabird Wreck in the Irish Sea, Autumn, 1969*, Natural Environment Research Council, Series C (4),, 14 (1971).
6 *Polychlorinated Biphenyls in the Environment* (US Department of Commerce, Washington, DC, 1972).

HARVEY AND STEINHAUER REPLY—We disagree with Longhurst and Radford[1] that our analytical results are incorrect. After several comparisons of the different methods, analytical standards and reproducibility ($\pm$ 20%) we are quite certain that our data are correct as they stand. It is relevant that during 1971–72, when about 30 ng l⁻¹ PCB was being measured we were also measuring 1 ng l⁻¹ or less in tapwater and groundwater. Also, in 1973–74 we measured PCB concentrations of 30–300 ng l⁻¹ in polluted estuaries near Boston, USA, at a time when North Atlantic values had declined to 0.8–2.0 ng l⁻¹. Clearly, the methods were capable of determining PCB concentrations present in different water masses over a wide range of values during the period in question.

We now agree, however, that our previous extrapolation was not justified. In the light of some recent data it seems that each area of the North Atlantic (the central gyre, doldrums, trades, and so on) is characterised by clusters of similar PCB concentrations which roughly correlate with salinity, wind direction and latitude[2]. As we do not have data from north of 45° N since 1972, nor from south of 20° N before 1973, our extrapolation from an average concentration to the

Table 1 Global production (t × 10⁴) of PCB, assuming US production to be half of global production[6]

1960	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971
3.44	3.32	3.48	4.06	4.62	5.50	5.90	6.84	7.52	6.94	7.72	3.66

of the magnitude reported by Harvey et al. could only be achieved if the total world production of PCB were put continuously into the North Atlantic.

For a more reasonable explanation for this result, two possibilities should be considered: first, that the analytical

1971–72 and 1973–74 data comprise regional clusters of stations, each cluster being widely separated from others, so that there was little geographical correlation within or between the two sets of data. Even the two sets of data, one in each period, derived from the work of

entire North Atlantic was not warranted. In contrast, PCB concentrations have been measured regularly in the North American Basin during 1972–75. As this is the closest ocean basin downwind of the US industrial complex the large decrease observed there between 1972 and 1973 may only reflect changes in the North American PCB input and may have been the most rapid in the North Atlantic.

We intend to measure PCB concentrations north of 50°N during 1975 in waters more within the influence of European PCB losses.

Woods Hole Oceanographic Institution

[1] Longhurst, A. R., and Radford, P. J., *Nature*, 256, 239 (1975).
[2] Harvey, G. R., and Steinhauer, W. G., *Proc. second int.Conf. Environ. Biogeochem.* (in the press).

Avian vision

IN an article[1] on the visual control of head movements by walking birds, Friedman quotes me as maintaining that a bird's head moves backwards as well as forwards during walking. This is not so. In 1931 observation of hens had shown me that, as Friedman has proved for pigeons, the head of a walking hen is thrust forwards as rapidly as possible and then remains stationary while the body of the bird catches up. It seemed to me quite clear that this enables the hen to have a succession of sharply defined pictures of its environment rather than the continuously slightly fuzzy picture which would be obtained from steadily moving eyes.

Humans circumvent this problem differently. They fix visually upon a succession of points for varying times according to their momentary interest, and use a reflex capacity to control eye positions to compensate for bodily movement.

Most birds have a much larger area of high definition than humans, backed by a much less versatile data processor, and may find it advantageous to attend only to those objects in their environment that are moving independently. These can be distinguished from a complex background only when the eyes are stationary with respect to that background and are not confused by the apparent backward displacement of the nearer objects which will occur as a result of parallax while the eyes are moving.

In my article[2] I was not concerned at all with the head movements of walking birds but with the frequent up and down movements of the heads of stationary birds. This, I suggested would not only give feeding birds

longer ranges of vision—which it by no means always does—but would enable birds to use parallax to obtain a three-dimensional picture of their surroundings.

Binocular stereopsis is available to most birds, with their sideways-looking eyes, only over a very narrow field of forward vision, and with an extremely short 'baseline' between the eyes. Observation of the changes of the retinal image as a bird raises and lowers its head, however, provides the bird with a much longer baseline and gives the possibility of three-dimensional vision over the whole field of view of both eyes—often nearly 360°.

J. H. FREMLIN

*Department of Physics,
University of Birmingham,
B15 2TT, UK*

[1] Friedman, M B., *Nature*, 255, 67–69 (1975).
[2] Fremlin, J. H., *New Scientist*, 56, 26–28 (1972).

Plume spacing and source

SINCE the average distance between island-arc volcanic centres (~70 km) is approximately equal to the depth to the magma source (~100 km), Lingenfelter and Schubert[1,2] have suggested that the typical distance between hot spots (~3,000 km) is approximately equal to the depth to their source. Thus, they infer that the region of instability giving rise to the initially solid plumes of mantle material is near the core–mantle boundary. Certain fluid dynamic results may show this inference to be untenable.

In the case of island-arc volcanism[3], andesitic magma is generated along the Benioff Zone at a depth of about 100 km. The magma is much less dense than the overlying peridotitic mantle material and this two layer system, andesitic magma beneath peridotitic mantle, is gravitationally unstable. This situation is analogous to that of a layer of oil underlying a layer of water, and represents a type of fluid instability commonly known as Rayleigh–Taylor instability[4–6].

When perturbed from equilibrium, the less dense, lower fluid penetrates the overlying fluid at discrete and, ideally, evenly spaced points. This is the case in both two and three dimensions[4,5]. The characteristic wavelength of instability is dependent on the depth to the source layer (or thickness of the upper layer) only if the thickness of the source layer is greater than one tenth of the depth to the source[7]. That is, once the upper layer is 10 times thicker than the lower buoyant layer the dominant wavelength of instability is independent of the depth to the buoyant layer. In island arcs, judging from the

exceedingly small quantity of lava observed at the surface, the thickness of the magmatic layer is, surely, much less than 10 km. For those cases, the spacing of the volcanic centres is given by[4]

$$\lambda = (2\pi h_2/2.15)(\eta_1/\eta_2)^{1/3}, \text{ for } h_1 > 10h_2$$

where: λ = the dominant wavelength or spacing of volcanic centres; h_1 = the thickness of higher density layer; h_2 = the thickness of lower density layer; η_1 = the viscosity of denser layer (mantle); η_2 = the viscosity of lighter layer (andesitic magma).

By estimating roughly the contrast in viscosities ($\eta_1/\eta_2 = 10^9$), and from the observed spacing of the volcanic centres (~70 km), the magma layer in the case shown was probably a few tens of metres thick at the time of instability.

In the case of plumes, assuming, as did Lingenfelter and Schubert[1], that the process of instability is similar to that operative in island arcs, if the source layer is near the core–mantle boundary it must be greater than about 300 km thick for the plume spacing to reflect the depth to the source (that is, 3,000 km). Seismic evidence seems to indicate that a low density layer of this thickness cannot be present in the lowermost mantle. On the other hand, if it is assumed that the depth to the buoyant layer, wherever it is, is greater than 10 times the thickness of the buoyant layer itself, the thickness of that layer can be calculated from the relationship given already. For $\lambda = 3,000$ km and $\eta_1/\eta_2 = 10^3$, the buoyant layer thickness is about 100 km, whereas for $\eta_1/\eta_2 = 10^6$ it is about 10 km. A layer 10–100 km thick could be located seismically. Since the existence of a low density layer is a necessary condition for this type of fluid instability, which seems inherent in the plume theory as envisaged by Morgan, it is of paramount importance to ascertain its presence.

In conclusion, the distance between island-arc volcanic centres tells us little about the depth to the magma source and this is also probably true for the spacing between plumes if they arise from a Rayleigh–Taylor type fluid instability.

BRUCE D. MARSH

*Department of Earth and Planetary Sciences,
The John Hopkins University,
Baltimore, Maryland, 21218*

[1] Lingenfelter, R. E., and Schubert, G, *Nature*, 249 820 (1974).
[2] Lingenfelter, R E, and Schubert, G., *Bull volcan.* (in the press).
[3] Marsh, B. D., and Carmichael, I. S. E., *J. geophys Res*, 79, 1196–1206 (1974).
[4] Selig, F, *Geophysics*, XXX, 633 (1965).
[5] Biot, M. A., *Geophysics*, XXXI, 53 (1966).
[6] Whitehead, J A, Jr, and Luther, D S., *J. geophys. Res*, 80, 705–717 (1975).
[7] Biot, M. A., and Odé, H., *Geophysics*, XXX, 213, (1965).

reviews

LIND'S *Studies in Pre-Vesalian Anatomy** is a massive work; a stout quarto of more than 300 double-columned pages, which has been meticulously researched in libraries and archives in Europe and America. The fruits of Professor Lind's labours of the past 12 years are abundant. We are given 16th century documents—wills, letters, university and municipal records —which previously have never been published. We are given biobibliographies of eight pre-Vesalian anatomists. Finally, Lind provides elaborately annotated translations of six anatomical treatises originally published between 1497 and 1559. The result is a wonderfully full picture of the state of anatomy in the half century before Andreas Vesalius' *De humani corporis fabrica* (1543) transformed the subject. While Vesalius' genius is actually thrown into relief by comparison with his contemporaries, Lind's study documents the rich and vibrant anatomical tradition which preceded the *Fabrica*. Vesalius did not emerge from a vacuum.

With two exceptions, all of these pre-Vesalian anatomists were Italian, a fact which underscores the importance of the Italian universities in 16th century medicine. One of the exceptions was Andrés de Laguna (1499–1560), a peripatetic Spaniard who spent nine years in Italy. De Laguna wrote more than 30 books, including the *Anatomica methodus* (1535) which Lind has translated, though this is perhaps the least original of the texts which he has chosen. Though de Laguna himself was a figure of considerable fame, his *Anatomica methodus* was inferior to various similar works which also preceded Vesalius. One of these was Niccolò Massa's *Liber introductorius anatomiae* (written by 1536 though dated 1559 on the title page), also translated in the present volume. Educated at Padua, Massa (1499–1569) was an active physician who practised in his native city of Venice and who wrote widely on plague and syphilis. Whereas de Laguna returned solely to the ancients for authority, Massa was aware of the tradition of anatomical study in the west,

Groundwork for a transformation

that had begun with Mundinus (1275?–1326). Mundinus was also revered by Berengario da Carpi (1460–1530), whose *Commentary on Mundinus* (1521) is summarised in this volume. Lind published an annotated translation of Berengario's more famous *Short Introduction to Anatomy* (the *Isagogae breves* of 1522) in 1959.

Berengario was one of a group of several outstanding anatomists who were prominent between the time of Mundinus and Vesalius : a group which included Gabriele Zerbi (1485–1569), Alessandro Achillini (1463–1512) and Alessandro Benedetti (1450?–1512). Lind has translated Achillini's *Annotationes anatomicae* (1520) and Benedetti's *Anatomice* (1502). Lind's work is rounded off with translations of Johannes Dryander's famous, short *Anatomia capitis humani* (1536) and Giovanni Battista Canano's equally familiar *Musculorum humani corporis picturata dissectio* (1541?). The well known illustrations of these latter two works are also reproduced.

From this summary the richness of Lind's work should be apparent. Lind provides the material necessary to any consideration of the development of anatomy between about 1500 and 1540. His annotations point back to the sources—classical, Arabic, and contemporary—of these pre-Vesalian anatomists He notes the philosophical preoccupation of most of these figures, several of whom were equally at home in the world of 16th century Aristotelianism In fact, part of Vesalius' suc-

cess resulted from the single mindedness with which he pursued anatomy; many of his talented contemporaries lost depth because of their eclectic and catholic enthusiasms; the anatomical treatises which Lind has translated generally present only a fraction of those anatomists' total outputs. His translations are lucid without being anachronistic. Where appropriate, he gives modern anatomical equivalents in parentheses, but he never tries to make these texts appear more modern than they really are. The short, introductory biographical studies of his subjects generally contain new material and full assessments of modern secondary literature.

In sum, this fine work tells us a great deal about the anatomical milieu in which Vesalius worked. It helps us see Vesalius' undoubted genius in clearer perspective. Though Professor Lind's bibliographic enthusiasm will appeal only to a handful of specialists, the work as a whole deserves a wide audience. Together with his earlier translations of Berengario and of Vesalius' *Epitome,* it permits the Latinless reader to appreciate for himself the development of anatomy in the first half of the 16th century. Translation and annotation may not be the most exciting part of scholarship, but they can be most exacting, and Professor Lind possesses the tenacity and erudition of the ideal translator. At $18.00 his latest book is an unusual bargain at a time when book bargains are few and far between. **W. F. Bynum**

Drug effects

Handbook of Drug and Chemical Stimulation of the Brain: Behavioural, Pharmacological and Physiological Aspects. By R. D. Myers. Pp. xvi+759. (Van Nostrand Reinhold: New York and London; Medical Economics: Oradell, New Jersey; February 1975.) £19.90.

TECHNIQUES for the direct administration of drugs into the cerebrospinal fluid, or into discrete areas of the central nervous system (CNS) have been in use for some time and have proved increasingly popular. The apparent directness and disarming simplicity of this approach to the examination of the CNS pharmacology of various bodily functions or behavioural processes has been particularly attractive to many research workers in the fields of physiological psychology and neuroendocrinology. This volume describes the many practical difficulties and pitfalls associated with this approach.

The main advantages of the direct administration of substances into the CNS are, first, that one may examine the effects of compounds that do not readily penetrate the blood–brain barrier after systemic administration and, second, that by localised applications one may hope to localise a particular drug action to some particular neuronal system in the CNS. The approach suffers, however,

from severe practical limitations. Unless very small volumes of fluid are injected, the spread of substances may be far too great to allow localisation. It is also difficult to judge what an appropriate dose of injected material should be. If compounds that have highly selective pharmacological actions on peripheral organs are injected in high local concentrations into the CNS, the observed effects may result from the non-specific toxic or membrane stabilising effects commonly found in many drugs. The absolute specificity of action of any drug is a myth that many of those engaged in research in this area have found beguiling.

In spite of the practical and theoretical difficulties, there is little doubt that research of this type has many useful applications. Although sophisticated methods exist for the micro-application of drugs and other substances onto single neurones in the CNS by micro-iontophoresis, one could only expect to elicit observable changes in behaviour or physiological functions after the exposure of much wider populations of neurones to drug stimulation or inhibition. Dr Myers is an acknowledged authority in this area and he has prepared a useful and scholarly compendium of the literature. He discusses in detail the principles and experimental methods in current use, and reviews the results obtained in attempts to manipulate a wide variety of bodily functions or behavioural states, including the control by the CNS of cardiovascular and respiratory functions, neuroendocrine relationships, sexual behaviour, body temperature, hunger, thirst, sleep, arousal, pain and cognitive functions. Each topic is dealt with in sufficient detail to justify the use of the term 'Handbook' in the title of the volume.

The book is amply illustrated, and each section has a "Master Table" summarising the available results. It is also liberally documented, with more than 1,400 references. Unfortunately, its value is diminished greatly by the fact that references cover the literature in detail only up to early 1972; this results in what seems to be a curiously dated view on some issues. For example, the evidence that cholinergic mechanisms play a rôle in learning and memory is reviewed in some detail, whereas the rôle of monoamines is dealt with in half a page. The latter is one area in which much has happened since 1972.

Dr Myers has presented facts and has not, to any extent, aimed to integrate psychological, pharmacological and physiological findings.

The author has produced the first detailed survey of an active and often poorly understood area. The volume will certainly be a useful reference work for those interested in this field.

S. D. Iversen

Drugs and Behaviour: A Primer in Neuropsychopharmacology. By Ernest L. Abel. Pp. ix+229. (Wiley-Interscience: New York and London, October 1974.) £7.55.

THE word 'primer' was originally used at the time of the Reformation to describe a prayer book or devotional manual for the use of the laity. Only later did the term acquire its common connotations of inky schoolroom, and the plain child's introduction to simplified truths.

A primer in neuropsychopharmacology might be expected to fulfil something of both these useful functions. The members of the laity who are not specialists in this particular science will be grateful for a reliable and intelligible guide to esoteric mysteries; and those who are in the graduate schoolroom, beginning the specialist study of this subject will be grateful for an accurate professional introduction.

At first glance this book has much to recommend it under both headings. It is eminently readable, and its author has natural gifts as a master of exposition. Illustrative examples are well chosen to exemplify general scientific principles—hunger as an adrenergic mechanism, the cholinergic mediation of water intake, serotenergic mechanisms and sexual behaviour. Headings and sub-headings order the matter well. Graphs are introduced at the apt moment, and are clear in the messages they carry. All in all, the immediate impression is of a simple textbook of considerable substance and expert presentation.

Closer reading brings some disappointments. The 'psycho' element in the neuropsychopharmacology (a truly Germanic word), receives scant treatment. The word 'placebo' is not to be found in the index, and nowhere does the influence of set and expectation receive adequate attention.

And as one gets deeper into the text so many errors and ommissions come to light that one begins to feel that the book has been contrived as basis for a game of 'spot the deliberate mistake'. THC is described as "the" active material in cannabis. The picture of amphetamine psychosis remains undescribed and classical work on the matter is ignored and unreferenced. The description given of schizophrenia would make Bleuter turn in his grave, and is not only misleading but damagingly misleading. The sharp differentiation drawn between psychological and physical dependence must be described as nonsense.

In short, the author should be encouraged to produce as a second edition of this readable and essentially worthwhile book (which ought to reach many editions) a text which will neither mislead the laity nor misinform the schoolroom. Otherwise anathema, and a black mark.

Griffith Edwards

Generally animals . . .

Animal Physiology: Adaptation and Environment. By Knut Schmidt-Nielsen. Pp. xvi+699. (Cambridge University Press: London, March 1975.) £7.25.

No animal exists, or can exist, independently of an environment. It may logically be argued, therefore, that all physiology is environmental physiology, for physiology is concerned with the functions of living organisms. The book under review deals at an elementary level with the familiar subjects of physiology, such as respiration, circulation, digestion and so on. Its 13 chapters are grouped according to major environmental factors: oxygen; food and energy; temperature; and water. Finally, movement, information, and integration are discussed, and it is shown how these functions are correlated and controlled in the living organism. There are six appendices and a comprehensive index. Although published by the syndics of the Cambridge University Press, the book has been printed in the United States of America and uses trans-Atlantic conventions and spelling.

As in his earlier books, the Professor of Physiology at Duke University writes with a clarity that imparts beguiling simplicity to quite complex concepts. He states in the preface that his latest effort was written in "anger and frustration" because he was unable to give his students a book which, in simple words, says what he finds "exciting and important in animal physiology, that deals with problems and their solutions, that tells how things work". He has succeeded in producing just such a book, and it will doubtless satisfy quite precisely the requirements of American undergraduate syllabuses. As far as the British market is concerned, however, I fear that the level may be a trifle too elementary.

The field of environmental physiology is now so vast that not even this large text-book can avoid being somewhat superficial. There are plenty of exciting ideas, but they are discussed too simply, and their documentation is rather inadequate. The selection of references, which "vary from brief and simple essays to large, comprehensive treatises", seems arbitrary and erratic. In places, too, there are signs of untoward haste in the writing; for example: "The degree of heating that a lizard can attain by heating up in the sun can be spectacular" (p. 358); and: "For aquatic insects the problem is to eliminate excess water and, as was mentioned above, this problem is usually handled by the kidney or equivalent excretory organ" (p. 418–9).

It is easy to cavil at the efforts of others: and the wider an author's vision, the easier it becomes to criticise his work on points of detail. There are, naturally, many aspects both of fact and of emphasis, with which other physiologists and zoologists may disagree—but this should not blind them either to the magnitude of Schmidt-Nielsen's conception or to the skill with which he has realised it. **J. L. Cloudsley-Thompson**

Zebrasoma, the sail-finned surgeon fish. From *The Guinness Guide to Underwater Life.* By Christian Petron and Jean-Bernard Lozet. 224 pages including over 100 colour illustrations and 280 in black and white. (Guinness Superlatives: Enfield, April 1975.) £6.95.

. . . particularly men

Physiology of the Human Body. By J. Robert McClintic. Pp. xxvii+588. (Wiley: New York and London, January 1975.) £6.80.

A MAJOR difficulty confronting any reviewer of this book is that of indicating clearly the type of reader to whom it should appeal, especially as the author states that it is directed at "all levels of reader" including those "planning to enter a health-orientated profession".

Among those professions, however, medicine is not specifically mentioned, and, in fact, the book is not one which could be recommended to a medical student as his working text since too many topics are treated superficially without sufficient attention to experimental evidence. The author has, however, produced a masterpiece of didactic compression which is commendably free from serious inaccuracies.

The text is liberally embellished with exceptionally clear tables and figures, often in the multicolour format familiar to readers of *Scientific American,* to which frequent reference is made in the bibliography. A most valuable feature of the book is a series of foldover plates depicting the structure of the human body layer by layer, and some might think that a study of these could save a great deal of time commonly spent in the dissecting room.

So much information is crammed into some 550 pages that redundancy is minimal and, therefore, great concentration is demanded of the reader. To aid him in monitoring his progress, however, each chapter concludes with a summary and a set of questions. Much attention is given to diseased states (presumably in the interest of "relevance") but the wisdom of including a thumb-nail sketch of the symptomatology of acute appendicitis is debatable. Likewise, an attempt to summarise biochemistry in a 12 page appendix, composed almost entirely of structural formulae, seems ill-judged, as does the inclusion of an elaborate table listing the symptoms of drug abuse and providing a key to junky slang; and the glossary at the end of the book usurps the function of the lexicographer by including such definitions as "quality—the nature and characteristic(s) of something". Misprints are rare but an eye-catching example amongst the questions on the digestive tract reads as "Discuss the importance of the lover in body function".

The growth in popularity of courses in human biology in both schools and universities may well ensure for this book a large market. **R. V. Coxon**
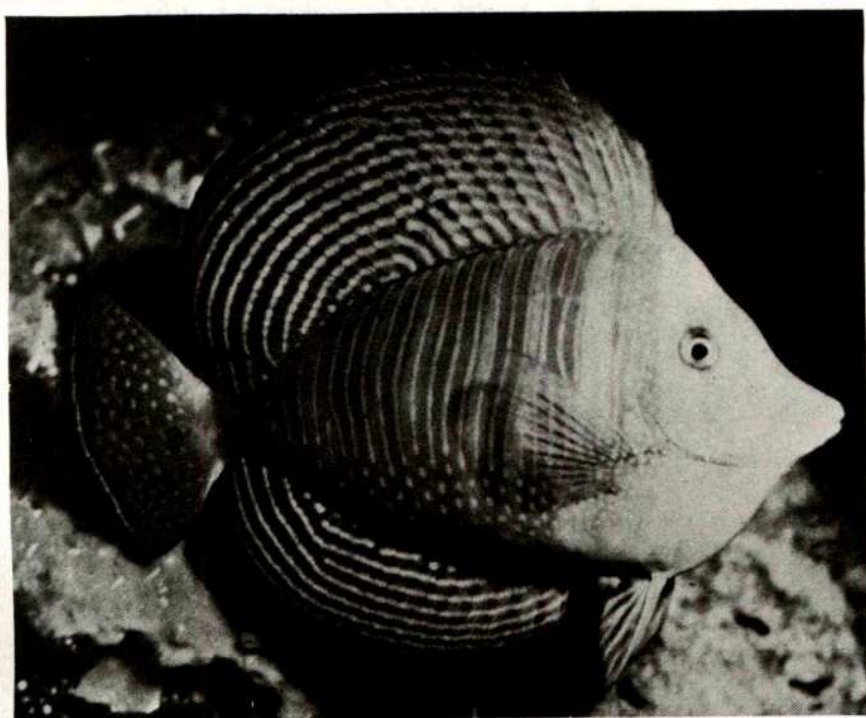
Mathematical tools

The Mathematics of Diffusion. Second edition. By J. Crank. Pp. viii+414. (Clarendon: Oxford; Oxford University Press: London, March 1975.) £12.50.

THIS volume could be regarded more as a tool kit than a book. Most of the content comprises a collection of mathematical methods for the analysis of diffusion problems, together with the solutions of the appropriate equations for many common situations. As mathematical results tend to be less transient than those of most other sciences, the bulk of the book is unchanged from the first (1955) edition, save for the addition of new references. In this new edition there are many cross-references to its alter-ego *The Conduction of Heat in Solids* by Carslaw and Jaeger; the latter volume presents a wider range of solutions to the diffusion equations and Crank has chosen those which are most appropriate to diffusion problems.

New material in this edition includes a chapter on non-Fickian diffusion, which provides an interesting review of solvent diffusion in glassy polymers, but it is somewhat out of place in this book in that it is inconclusive. A new chapter on diffusion in heterogeneous media is particularly timely as composite materials have become so widely used. The chapter

on numerical methods has been extensively revised to allow for the dramatic effects of developments in digital computers. Sadly, however, several diagrams of quaint analogue calculating machines have been lost in the process. This chapter is too brief, given that it is now often easier to compute a numerical solution than to derive the analytical solution, if one is not too proud. The challenge to a book of this kind is to provide enough detail to allow the average user to write the programme without consulting other sources. Crank dodges this challenge and simply gives a well referenced outline of the available methods.

This volume is a worthy successor to the first edition and will undoubtedly become an indispensable reference source for all who are concerned with diffusion problems.

P. D. Calvert and N. C. Billingham

Dynamic patterns

Dynamic Patterns of Brain Cell Assemblies. By A. K. Katchalsky, V. Rowland and R. Blumenthal. Pp. 187+viii. (MIT Press: Cambridge, Massachusetts, and London, 1974.) $15.00; £7.50.

WHAT is meant by "dynamic patterns"? In the present context the expression implies the idea that systems are made up of a complex hierarchy of smaller and larger "flow patterns" in which "things" are self-maintaining features of the flows. The concept also implies that the flow patterns can undergo sudden flip-over alterations, or translations, to new stable self-maintaining arrangements. Thus, one could say that the world is made up of complexes of movements, or dynamic patterns.

How important are these dynamic patterns? Are they merely biomathematicians' fantasies—intellectual toys—or are they of fundamental significance in biology? This book reports on a Neurosciences Research Programme Work Session, held in 1972, when various neuroscientists came together to consider the phenomenology of dynamic patterns and their relevance to neurobiology. Examples of dynamic patterns are presented from many fields including biology—the cellular structure that develops in a heated dish of spermaceti oil, self-maintaining cloud patterns, the well known Beloussov-Zhabotinsky reaction, and so on. Indeed, the invocation of dynamic patterns, or "cooperative non-equilibrium phenomena" is fashionable in fields as diverse as molecular interactions, developmental biology and sociology. My feeling is that these phenomena are likely to be of great significance, particularly for developmental biology and neurophysiology, and this book provides a useful introduction for non-specialists.

R. M. Gaze

Global tectonics and fossil fuel

Petroleum and Global Tectonics. Edited by Alfred G. Fischer and Sheldon Judson. Pp. xii+322. (Princeton University Press: Princeton and London, May 1975.) Cloth, £8.70; paper, £4.45.

AT the present time the surest way to get any geological manuscript into print is to postscript the title ' . . . and Global Tectonics'.

This volume, *Petroleum and Global Tectonics,* contains nine papers presented at a conference held in 1972 to honour Hollis D. Hedberg of Princeton University. The papers cover plate tectonics (Bullard), heat flow and vertical movements of the crust (Morgan), the origin and growth of basins (Fischer), rift valley basins and the sedimentary history of trailing continental margins (Kinsman), the petroleum and plate tectonics of the southern Red Sea (EXXON), marine sediments, geosynclines and orogeny (Curray), geochemical formation of oil (Erdman), geothermal gradients, heat flow, and hydrocarbon recovery (Klemme), and the distribution and geological characteristics of giant oil fields (Moody).

The idea of choosing a conference theme of interest to both academic and industrial geologists is highly commendable. But the academic and industrial authors seem to have been mutually embarrassed and to have been uneasy about the theme. Sir Edward Bullard thought the impact of plate tectonics on petroleum exploration was "rather weak" (p. 17), and Moody was similarly perplexed: 'I have some difficulty in trying to relate giant oil fields to the "New Global Tectonics" *per se'* (p. 314).

The net result is that most authors have gone off and 'done their own thing', either writing about global tectonics or about petroleum, but seldom about both.

It is only in the papers by the Exxon group, Klemme and Moody, that the two topics of petroleum and global tectonics are actually integrated, and very successfully so. The material for these three contributions is largely taken from back numbers of the *Oil and Gas Journal* and from the *Bulletins* and *Memoirs* of the *American Association of Petroleum Geologists.*

The volume does include, however, several excellent papers on sedimentary basins, geothermal gradients, modern marine sedimentary environments, rift valleys and the origin of oil.

The book is attractively produced and the illustrations are ample and clear.

R. C. Selley

obituary

Constantinos Doxiadis, known for his planning projects in a number of developing countries, has died in Athens at the age of 62.

Born in Bulgaria, he studied architecture at Athens Polytechnic and obtained a doctorate in engineering at Charlottenburg Technical University, Beira. He was director of the Athens Technological Institute and founder of the World Society of Ekistics, a term, coined by him to describe the interdisciplinary science of human settlements. He promoted a unique and valuable series of conferences on town planning studies, held annually in Athens and the Greek islands during the past dozen or so summers. To these Delos symposia—probably Doxiadis' primary contribution to his field—he attracted many leading exponents of different disciplines, who would not otherwise have had the opportunity to exchange ideas. Doxiadis was, at the time of his death, preparing a number of documents for the United Nations conference on human settlement, to be held at Vancouver in 1976.

Elizabeth L. Hazen, the coinventor of Nystatin, the first antibiotic successful in combating human fungal diseases, died in Seattle at the age of 89.

The greater part of Dr Hazen's career was spent with the state Health Department's division of laboratories and research. She and her coworker there, Dr Rachel F. Brown, screened hundreds of soil samples in the search for new antibiotics, finally tracing a microorganism that produced the antibiotic which became known as Nystatin. She received her doctorate in bacteriology from Columbia University, after graduating from the State College for Women in Columbia, Mississippi, and also served as an investigator in the department of dermatology at Columbia University College of Physicians and Surgeons.

Sir Geoffrey Ingram Taylor, a mathematician who made valuable contributions to engineering and geophysics, has died at the age of 89.

Sir Geoffrey had mainly been concerned with abstract hydrodynamical themes and later progressed to research in the practical problems of geophysics and meteorology. He was formerly Yarrow Research Professor of the Royal Society, of which he became a fellow in 1919. In 1913, he was meteorologist to the Scotia Expedition to the North Atlantic, and in France during World War I, he served as meteorologist to the RFC. He resumed this appointment during World War II and studied the motions of the air, and the cause and effect of eddies —many of these problems were applicable to aircraft. He was also involved in the design of better parachutes. He worked with the group which caused the first nuclear explosion at Los Alamos, New Mexico in 1944 and in that year he was knighted. Later in 1944, he won the Copley Medal, the senior award of the Royal Society, for contributions to aerodynamics and the structure of metals.

Joseph Proudman, CBE, FRS, the distinguished mathematician and oceanographer, has died at the age of 86.

Professor Proudman first studied the dynamics of tides at Trinity, Cambridge, and this was to become his main scientific interest. He returned to Liverpool, where he had taken his first degree, as a lecturer in 1913, and was appointed the first professor of applied mathematics in 1919. In 1933 he transferred to the chair of oceanography, which he held until his retirement in 1954. In 1916 Horace Lamb asked Proudman to assist him in preparing a report for the British Association on the state of research on ocean tides. This led him to found the Tidal Institute (now Bidston Laboratory of the Institute of Oceanographic Studies), concerned with all aspects of tides. The Institute acquired an international reputation for its tidal prediction services as well as for fundamental research. He was pro-vice-chancellor of Liverpool from 1940–46. He was a fellow of the Royal Society, which awarded him the Hughes Medal in 1957. He acted as secretary of the International Association of Physical Oceanography and as president from 1951–54.

announcements

Award

The Canadian Public Health Association has awarded the 1975 **ORTHO Award** to **J. E. F. Hastings** for his research in health services, planning and education.

Appointments

The National Environmental Research Council has appointed **A. W. Woodland** as director of the Institute of Geological Sciences.

Michael Sela has been elected president of the Weizmann Institute of Science in Rehovet, Israel.

International meetings

September 1–2, **Molecular beam kinetics,** Heriot-Watt University, Edinburgh (Dr A. R. Burgess, Department of Chemical Enginering, University College London, Torrington Place, London WC1E 7JE, UK).

September 1–3, **Thermodynamical properties of gravitational fields,** Newtown, mid-Wales (B. F. Schultz, Department of Applied Mathematics and Astronomy, University College, PO Box 78, Cardiff CF1 1XL, UK).

September 1–3, **Industrial crystallisation,** Usti Nad Labem, Czechoslovakia (Dr Sci Ing Nyvlt, Research Institute of Inorganic Chemistry, Revoluni 86, CS400, 60 Usti Nad Labem, Czechoslovakia).

September 1–4, **Electronic properties of solids under high pressure,** Louvain, Belgium (Luan Gervan, Laboratories of Solid State Physics, Department of Physics, Celestunenlaan 200D, B30/0, Leuven, Belgium).

September 1–5, **Charles Lyell centenary symposium,** London (J. C. Thackeray, Institute of Geological Sciences, Exhibition Road, London SW7 2DE, UK).

September 1–5, **Low energy electron diffraction and electron spectroscopy of solids,** Budapest, Hungary (Secretariat, LEED and Electron Spectroscopy of Solids Conference, Research Institute for Technical Physics, 1325 Budapest, PO Box 76, Hungary).

September 1–5, **Luminescence,** Tokyo, Japan (S. Shionoya, Institute of Solid State Physics, Tokyo University, 7221 Roppongi, Minato-ku, Tokyo 106, Japan).

September 1–5, **Combustion,** Orleans, France (Secretariat of the second European Symposium on Combustion, CRCCCHT–CNRS, Avenue de la Recherches Scientifique, CEDEX F45045, Orleans la Source, France).

September 1–5, **Computer education,** Marseille, France (AFCET, Immeuble Centre Dauphine, Place du Marechal de Lattre de Tassiguy, Paris 16e, France).

September 1–5, **Controlled fusion and plasma physics,** Lausanne, Switzerland (F. Hofmann, Centre de Recherches en Physique des Plasma, Ecole Polytechnique Federale de Lausanne, Avenue des Bains 21, CH1007 Lausanne, Switzerland).

September 1–5, **Drug abuse,** London (International Council on Alcohol and Addictions, Box 140, 1001 Lausanne, Switzerland).

September 1–5, **Mechanics in reactor technology,** London (Mr H. M. Carruthers, Associated Nuclear Services, 14–16 Regent Street, London SW1Y 4PH *or* British Nuclear Energy Society, 1–7 Great George Street, London SW1P 3AA, UK).

September 2–4, **Quantum electronics,** Oxford (The Meetings Officer, The Institute of Physics, 47 Belgrave Square, London SW1X 8QX, UK).

September 2–4, **Energy utilisation and conservation,** East Kilbride (Conference Organiser, Birniehill Institute, National Engineering Laboratory, East Kilbride, Glasgow G75 0QU, UK).

September 2–5, **Electronic circuit techniques,** Canterbury, UK (Conference Department, Institution of Electrical Engineers, Savoy Place, London WC2R 0BL, UK).

September 2–6, **Cell differentiation,** Copenhagen, Denmark (DIS Congress Services, 3 Knabrostraede, DK 1210 Copenhagen, Denmark).

September 2–6, **Neurochemistry,** Barcelona, Spain (Dr J. Sabater, Instituto de Bioquimica, Calle Roberto Bassas 1, Barcelona 14, Spain).

Person to Person

'Yeti' discovered in Bhutan

A mummy-like object found in the state of Bhutan in the Himalayas and photographed by Pushkar Singh, who believes that it is the remains of a 'yeti' or abominable snowman. He would be grateful for any comments from readers who may have come across such an object on their travels (82 Mehdipatnam Colony, Hyderabad 500028, Andhra Pradesh, India).

Disaster message. Research group invites voluntary involvement of people from all disciplines interested in improving disaster assessment and relief including possible short term overseas surveys of up to 3 months (London Technical Group, 41 Queen's Gate, London SW7, UK, tel. no. 01–589 9076 ext. 14).

Immunodeficiency – cancer. Any physicians who have treated persons with primary immunodeficiency diseases who have subsequently developed cancer or who know of such cancer cases, are encouraged to register them in an international immunodeficiency–cancer registry. Information on patients confidential, data from the registry available to all participants (B. D. Spector *or* J. H. Kersey, Immunodeficiency–Cancer Registry, Box 609 Mayo, University of Minnesota, Minneapolis, Minnesota 55455).

September 3–5, **Microtubules and microtubular inhibitors,** Beerse, Belgium (M. Borgers *or* M. de Brabander, Janssen Pharmaceuticals, Research Laboratories, 2340–Beerse, Belgium).

September 3–6, **Lipoproteins and hyperlipidemies,** Lisbon (Professor M. J. Halpern, Department of Biochemistry, Faculty of Medicine of Campo Santana, Lisbon, Portugal).

September 3–8, **Artificial intelligence,** Tbilisi, USSR (Professor E. Sandewall, General Chairman IJCAI–75, Artificial Intelligence Laboratory, 545 Technology Square, Cambridge, Massachusetts 02139).

September 4–9, **Stereology,** Gaithersburg, USA (Fourth International Congress for Stereology, Organising Committee, Dr G. A. Moore, National Bureau of Standards, 31203, Washington DC 20234).

September 7–9, **Conservation for decision makers,** Kinshasa, Zaire (International Union for the Conservation of Nature, 1110 Morges, Switzerland).

September 8–10, **Engineering and food quality,** Cambridge, UK (Dr R. Jowitt, National College of Food Technology, St George's Avenue, Weybridge, Surrey, UK *or* Gesselschaft Deutscher Chemiker — Geschaftsstelle D6000, Frankfurt, M90 Postfach 900440, German Federal Republic).

September 8–11, **Gas dynamics of explosions and reactive systems,** Orleans, France (Centre National de Recherche, Scientifique 45045, Orleans-Cedex, France).

September 8–11, **Electron microscopy and analysis,** Bristol, UK (Dr J. E. Steeds, H. H. Wills Physics Laboratory, University of Bristol, Bristol BS8 1TL, UK).

September 8–11, **Marine natural products,** Aberdeen, UK (Dr J. F. Gibson, The Chemical Society, Burlington House, London NW1V 0BN, UK).

Miscellaneous

Vision research. In celebrating their 350th anniversary, the Worshipful Company of Spectacle Makers proposes to award a limited number of research fellowships (up to £3,000 per annum for up to 3 years). Intended as personal support only—the recipient's university or place of research will be expected to provide working accommodation, apparatus and facilities.

nature

July 24, 1975

Let's hear it for the PhD

EIGHTEEN months ago the House of Commons Expenditure Committee issued a report on Postgraduate Education (House of Commons Paper No. 96). The general thrust of the document was that growth in postgraduate education had been insufficiently controlled, that postgraduate education should be shaped less by student demand and more by the needs of the economy and society as a whole, and that there should be a major change of emphasis towards postgraduate education being for those who had already experienced the world outside university. We thought at the time that it was a poor report as far as it applied to the PhD, and now that the House of Commons has just got round to debating it, we still think it's a poor report.

Much play was made, during the short debate, of the lack of any formal response by the Department of Education and Science to the document. By way of explanation the Under-Secretary (Miss Joan Lestor) pointed out, rather apologetically, that a large number of bodies were interested and it took time to collect and consider their views. Since the report is a potential threat to the whole way of doing academic research and since the bodies with an interest all have their offices within a bus-ride of the department one wonders why the delay in standing up for the PhD—could it be that the department is in broad agreement with the report? Miss Lestor's opaque speech gave no clues.

Mercifully, at the very end of the debate, one member, Dr Jeremy Bray, was prepared to stand up and plead for more understanding of the basic purposes of postgraduate education. "We need a permeation of the attack on the problems of modern society by the robustness, objectivity, and the exposure to the test of experience, which is the characteristic of good research. Any activity which fosters that and which seeks to spread those qualities within our society should not be lightly brushed aside." An unashamedly Popperian viewpoint probably lost on the House which immediately went on to discuss police recruitment.

The PhD degree is a relatively easy target to attack because its recipients do not seem to confer on society the same sort of benefits as, say, medical doctors. More recycle into the educational system than go elsewhere (not that industry, commerce and the Civil Service wants them all that much). Many get their degree and then seem to throw it all away by going on to do something apparently totally unrelated—an offence which is widely practised, indeed encouraged, after 16 years of education but totally unacceptable after 19. And they are alleged to be strangely unresponsive to society's needs, although they are invariably supervised and guided by more senior people.

What ought to be more widely known is that at least as far as the research student is concerned the transition from undergraduate to postgraduate work is such a change in style that few of those who drift into research for fear (as Mr Neil Marten put it) of the outside world survive long. To switch from a situation in which every problem has an answer (and preferably one that can be written down in a couple of sheets of examination paper) to a situation in which one is not even sure that the problem is worth asking, let alone whether it is answerable, is to expose oneself to a new and often icy world. And if it is done at all, it must be done young. The record of those who do come back for a research degree after several years in, say, industry does not make a compelling case for the post experience grant.

Many take the PhD degree but do not then seem to capitalise on it; this is easily seen as waste, and no doubt the Expenditure Committee could see it as that, but substantial numbers of students realistically face the fact that after three years of conducting research they are not cut out for a lifetime of it. In those three years, though, they will have learnt a whole new philosophy and methodology (and learnt by experience, not instruction). There are other places where such experience is needed than in the laboratory.

Finally, what should shape postgraduate education—the needs of the economy and society or student demand? The committee leaned to the former and there is much to be said for manpower planning in higher education. But society is generally only capable of servicing its present needs, and somewhere there has to be some very careful thinking about what the community will need thirty years hence. The future of a technological society is at least partly in the hands of the emerging student; to deny him the right to choose what sort of society he wants to work for is to try and prevent the further evolution of society—however reasonable the arguments on the other side may seem. □

Seeds of hope

The improved varieties of staple crops which hold out the eventual hope of feeding the world's growing population, will depend for some of their most valuable characteristics on the genetic raw material contained in the old indigenous varieties of crops cultivated by peasant farmers for thousands of years. But this pool of inherited variation is rapidly drying up because of the success of the very varieties it helped to create. The primitive 'landraces' and their wild relatives have in the past proved an invaluable source of resistance (worth many millions of pounds) to serious pests and diseases, and have provided genes for many other desirable characters such as improved nutritional quality and adaptations to drought or cold. The serious implications of the rapid disappearance of this reservoir of valuable genes is now recognised in many quarters, and work to conserve the variation which remains is under way throughout the world. Eleanor Lawrence reports on one of the first institutes specifically set up to tackle this problem as it enters its second decade.

THE Genetic Resources Unit established by the FAO within the Aegean Regional Agricultural Research Institute (ARARI) just outside Izmir, on Turkey's Aegean coast, started life in 1964 as an experiment unique at the time. It was the first such unit specifically set up to deal with the collection, storage and evaluation of the rapidly vanishing genetic variation in cultivated crops within a region holding a large part of the remaining stocks of primitive cultivars and wild relatives of some of the most important temperate crops —wheat, barley and grain and forage legumes.

The concept of a 'gene bank' itself was hardly new even in the early 1960s. Plant breeders had collected useful material from all over the world to incorporate into plant breeding trials, but material not of immediate use had often been discarded. Large reference collections of seed of important crops were held at the National Seed Storage Laboratory at Fort Collins in Colorado, and in the USSR, where the now irreplaceable collections made by N. I. Vavilov and his coworkers in the 1920s and '30s were kept in Leningrad and at other centres throughout the country. Collections of various types and sizes were also held throughout the world.
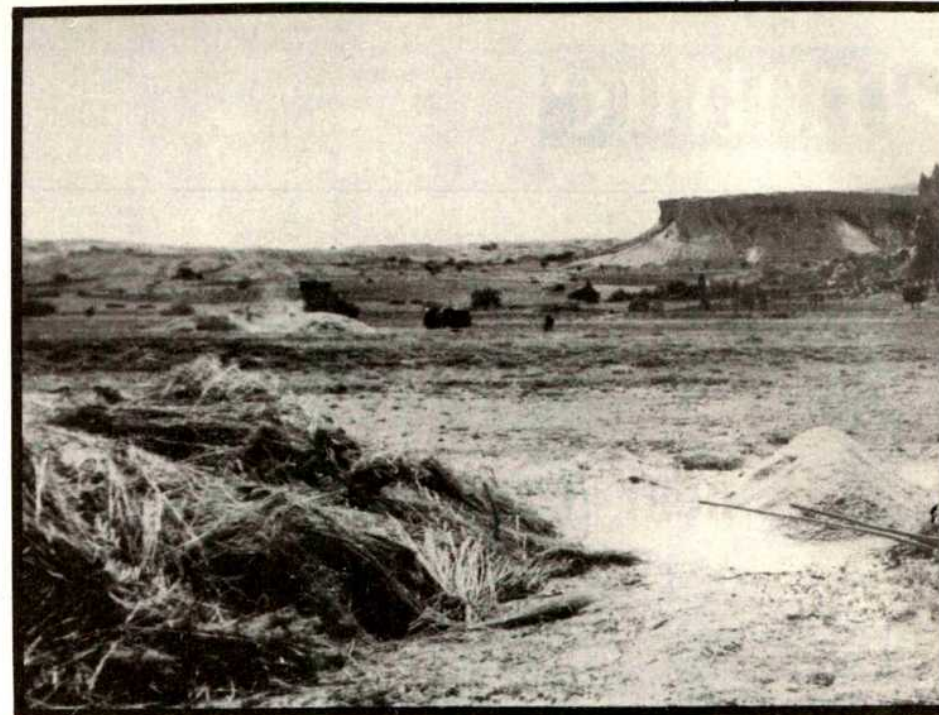
But there were as yet no medium-sized institutes, actually located in the countries where the greatest genetic diversity occurred, which had as their primary aim the systematic collection, and on-the-spot conservation and study, of valuable seed material, with the additional aim of screening and describing the material and making it freely accessible.

The importance of the Near East (stretching from Turkey to Pakistan in the east and Lebanon in the south) as a centre of diversity for many temperate crops stems from its complex geography and its long continuous history of agriculture. The Near East is the cradle of modern 'western' agriculture, based on wheat and barley as staples, which were first cultivated there some 10,000 years ago. Archaeological evidence shows that a mixed agriculture based on wheat and barley had developed throughout the region by the 8th century BC. Barley, emmer wheat (tetraploid *Triticum dicoccum*, a relative of the modern *durum* wheats), the modern hexaploid bread wheats (*T. aestivum*) and the diploid einkorn wheat (*T. monococcum*), still cultivated in parts of Turkey today, have all been found at archaeological sites dated between 6000 and 7000 BC.

But today, according to a survey carried out by FAO and the International Biological Programme published in 1973, improved varieties of both *durum* and *aestivum* wheat have very nearly replaced local cultivars except for remnants in the more remote regions. The wild progenitors of both wheat and barley can still be found and are even increasing in localities where they happen to be within protected 'national park' areas. Today wild barley flourishes in the ruins of Ephesus.

If this gene pool of inherited variation built up over thousands of years of evolution, selection and diversification into a wide range of habitats, is allowed to disappear completely, the plant breeder's ability to respond to a changing world situation will be severely restricted. These traditional 'landraces' and their wild relatives are often the only source of resistance genes to a wide variety of serious pests and diseases, such as stem rust in wheat. The old landraces are sometimes not very productive, and some do not respond to fertiliser treatment, but locally adapted populations can withstand conditions of drought or cold for instance which would soon lay low most modern varieties. These characteristics are needed to incorporate into breeding programmes with the generally more productive, modern varieties. Also, with fertilisers and pesticides growing more expensive each year, modern varieties which depend for their high yield on generous inputs of fertilisers may become less attractive. Traditional cultivars which produce good yields under less favourable conditions may prove a more rational basis for future breeding programmes
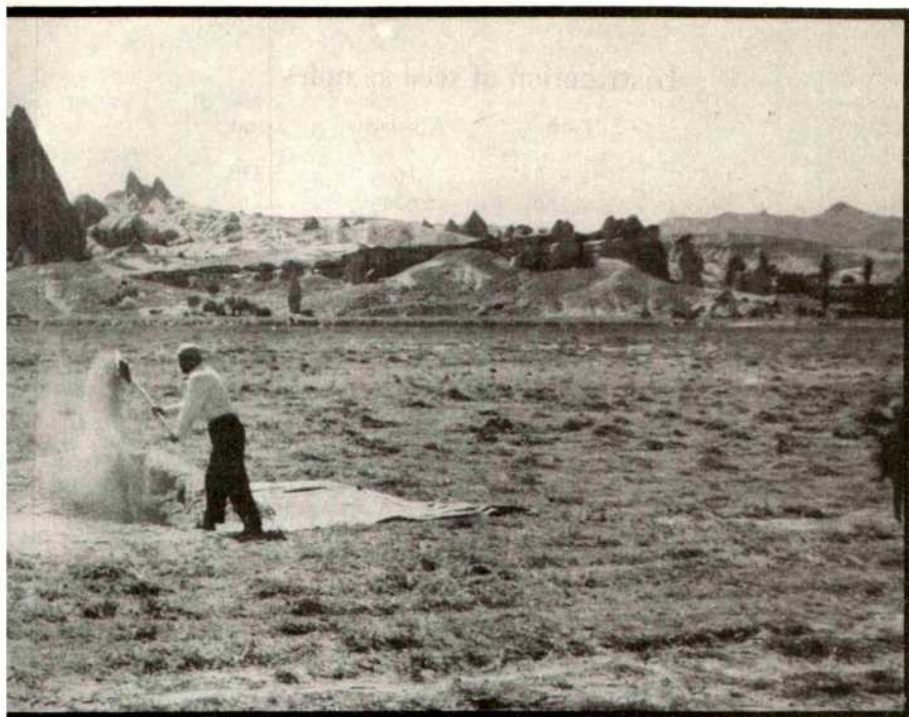
Harvest in Turkey: reservoir of genetic resources

for some areas.

In the decades since the Second World War, Turkey has seen widespread changes in agricultural practice. Changes in land use, introduction of improved varieties and modern methods of agriculture including the use of herbicides for weed clearance, have hastened the disappearance of the old locally-adapted populations.

In 1961, the Turkish government asked for international aid to research into problems posed by the changeover to modern systems of agriculture. In 1963 the United Nations Special Fund (now incorporated into the UN Development Programme) allocated funds and with FAO as the executive agency the 'Izmir Project' began. The Turkish request for aid coincided with FAO's own wish to establish a centre for the collection, study and distribution of forage legumes and grasses, and also with a growing consciousness of the urgent need to survey and collect rapidly disappearing germplasm of important crops. Over the nine years during which the project was financed by UNSF/UNDP, the Turkish government provided a total of $980,000 worth of land and staff salaries plus $110,000 in cash, and the UN around $1,290,000.

The original aim was to establish a Crop Research and Plant Introduction Centre, which as well as carrying out genetic resource surveys and conservation would maintain and initiate breeding programmes and also serve as the point of introduction for new varieties entering the country from abroad. The introduction of plant material from another country is always a risky operation because of the possibility of disease being brought in accidentally. Quarantine procedures for screening incoming material and certifying outgoing seed as disease free are an important part of the work of any centre involved with the distribution of plant material. It was also essential for a unit concerned with genetic resource conservation to keep a finger on the pulse of plant introduction so that they could foresee when the introduction of new varieties into previously undeveloped areas might threaten valuable sources of primitive cultivars.

But the early days of the project were dogged by organisational problems. At the time of its establishment, the Turkish government was also in the process of setting up its own agricultural research institute on the same site using the same funds and facilities provided for the FAO/UNSF programme. It proved logistically impossible to keep the two elements separate, and the original interpretation of the project by FAO as being one primarily of building an institute with an essentially international outlook for the collection, conservation and use of crop genetic resources became to some extent engulfed in the Turkish aim of building an institute of much wider and more immediate value to Turkish agriculture. This was recognised in a merger with the Ministry of Agriculture institute, the present ARARI, in 1967 and the present status of the genetic resources unit within ARARI. From 1970 until UNDP ended its financial aid in 1973, all UNDP/FAO funds were concentrated on the development of this discrete unit within the institute.

The original programme also proved to be overambitious given the limited financial resources and organisational difficulties. The present efficient and streamlined genetic resources unit has, for the time being, had to discard its original plans for full evaluation of the material collected and now concentrates on collection, storage, and computer documentation of the extensive herbarium and seed collections.

Since 1968, there has been a systematic collecting programme in crops particularly endangered by the changing patterns of agriculture. More than 2,600 samples of cereals, 3,759 samples of grain and forage legumes, 615 samples of vegetable and spice crops and 1,513 samples of 'industrial' crops (including over a thousand samples of opium poppy seeds) have been collected from all over Turkey; additional accessions have been received from other countries, and deposited in the excellent cold storage facilities completed in 1968.

The poppy seed collections were made in 1972, under the impending ban on opium poppy cultivation. No collections had been made previously so when the government ban was announced an emergency collection of seed stocks was mounted. Although FAO does not condone the traffic in opium, *Papaver somniferum* and *P. bracteum* are also important oil seed crops in more than 2,000 villages in Central Anatolia and it was hoped that varieties with high oil content and low morphine content could be selected for restocking. With the full cooperation of the government, local agricultural extension staff were quickly trained in the basic techniques of collecting and over 1,000 samples were gathered in the one season before cultivation stopped.

This exercise also proved an excellent example of the greater efficiency of collections organised and carried out by trained local staff, familiar with the terrain, language and customs of the country. The one-off expeditions mounted by foreign teams, unfamiliar with country and language can sometimes prove unproductive, and one of the roles of the Izmir centre has been to provide support and advice for the many expeditions which visit Turkey, attracted by the diversity of its natural and cultivated flora.

A survey and collection of the wild and traditional varieties of fruit and nut trees, such as almond, walnut, apple and peach, which were first cultivated in the Near East and grow wild in Turkey, has also been made. Although these are not staple crops, they are particularly vulnerable to changing land usage. The traditional mixed orchards where the old cultivars are grown are not being replaced, and large commercial plantations of new varieties are being planted instead. The wild species are also disappearing rapidly. Unlike herbaceous plants, tree germplasm is often difficult to conserve

as seed. A living collection assembled at Izmir was finally abandoned when it proved too difficult to maintain and evaluate the living trees in conditions far removed from those to which they were adapted, and attention was instead concentrated on selecting the best lines as a basis for varietal improvement. *In situ* preservation in protected areas probably gives the best chance of preserving the wild varieties.

The ending of UNDP aid in 1973 marked another change in emphasis for the Izmir centre. Since 1970 the international contributions had gone solely to the genetic resource unit, and this now continues with funds from the Swedish International Development Authority through FAO, which have been promised until mid-1976. At the same time, the scope of the genetic resource project was formally widened to take in the neighbouring countries of Syria, Iraq, Iran, Afghanistan and Pakistan, under the title of the Near East Project, which has now been in progress for one year, and aims, with Izmir as a focal point, to coordinate the national efforts in these countries, all important areas of genetic diversity.

The future role of Izmir as the main regional centre is now in some doubt, however, despite its excellent technical facilities, scientific record and core of highly trained, experienced Turkish staff. The problems arise from its present position within a government-controlled institute, where although funded internationally, it is still under the ultimate control of the director of the ARARI, and therefore not completely independent.

Financial support for the Near East Project after mid-1976 is to be channelled through the newly-established International Board for Plant Genetic Resources (IBPGR) which is considering an alternative for the regional centre to serve this programme. In the present reappraisal of world-wide genetic resource conservation at present being carried out by the IBPGR with a view to linking existing programmes into a global network, it would prefer where possible that the regional centres be autonomous and be able to take a truly international view of their essential function of making the seeds they collect and store freely available. The Consultative Group on International Agricultural Research, the parent body of the IBPGR, is setting up a new international agricultural research centre to serve the Near and Middle East in the Lebanon and this might prove a satisfactory location for the regional centre for genetic resource conservation as well, with Izmir continuing as a national centre —perhaps under international funding if necessary.

The Izmir project was a milestone in

Distribution of seed samples

	Turkey	Abroad	Total
1967	2,975	160	3,230
1968	214	2,624	3,214
1969	1,473	265	3,214
1970	3,668	195	1,738
1971	3,474	1,672	5,146
1972	2,186	976	3,062
1973	447	1,578	2,025

Over the years samples of about 75% of the collections have been sent overseas to scientists and plant breeders on request, with the greatest demand being for wheat, barley and grain legumes. Complete duplicate collections of the cereals have been sent to Canada, chickpeas to India, Holland and the Lebanon, lentils to Argentina and the Lebanon and broad beans to Sweden, East Germany, the United States and Australia. When seeds are sent they are always accompanied by requests that any information on performance and characteristics be returned so that records can be kept up to date.

the transition of genetic resource conservation from a state of individual effort into an internationally coordinated effort, but like many pioneer projects is now hampered by limitations imposed by its origin at a time when the problems of organising genetic resource work on such a scale were imperfectly appreciated.

Another candidate which has been put forward as an alternative regional centre is Ege University near Izmir.

There would be many advantages in locating a centre at Ege, which already has a good department of agriculture, existing collections and facilities including cold storage. Future work on genetic resources, once the initial urgent need for collection has been satisfied, can be usefully diversified into basic research in plant genetics, taxonomy, pathology and ecology which will illuminate the potential of the collected material for use in plant breeding programmes. The present FAO Project Officer feels that two projects especially would be particularly appropriate. As legumes always grow naturally in a symbiotic association with nitrogen-fixing rhizobia, which are specific for different species and probably as genetically variable as their plant hosts, a gene bank of rhizobia complementary to the legumes seems essential for the conservation of the natural symbiotic system, and would also prove invaluable for bacterial geneticists who are now trying to breed more efficient strains of rhizobia and attempting to extend their host range. The UN Environment Programme is considering plans at present for the international coordination of *Rhizobium* collections.

Such a centre would also be an ideal base for work in the variation found between different races of plant pathogenic fungi, which poses a problem for the breeder trying to incorporate resistance into the plant, and for surveys of the extent of disease resistance within areas where it has arisen naturally.

A completely new regional genetic resource centre, wherever it is eventually located, will, however, take some time to put into commission and time is in short supply as far as exploration and collection are concerned. The facilities at Izmir will certainly be needed more than ever in the next year or so. Most of the Near East countries have vigorous programmes of agricultural improvement, which within the next decade will further reduce the areas where the older varieties still remain.

Other changes in the Near East Project may be envisaged under future IBPGR funding. The anomalous position of the oil producers Iran and Iraq as recipients of international aid is unlikely to continue. Although the international organisations will be happy to provide expert help and training facilities for young scientists, it is hoped that Iran and Iraq can be persuaded to become contributors along with the other donor countries which comprise mainly the industrialised nations. Although scientists within these countries are fully aware of the necessity to conserve their remaining sources of variation in cultivated crops, it is sometimes difficult to persuade governments that such essentially long term projects should be supported. But the Izmir Project showed that for a comparatively modest sum, compared with the thousands of millions of dollars often spent on prestige technology, irreplaceable sources of variation can be safely banked for the future. □

international news

(MOS)MOSCOW,July 15 (AP)-Muscovites watch televised program Tuesday from Soviet Baikonur space center. Shown on screen is Alexei Leonov, commander of Soyuz space-ship as he enters Soyuz.(AP WIRE-PHOTO) (dm/stf/BY) 1975

THE Apollo-Soyuz link-up has undoubtedly proved a salutary exercise in the logistics and diplomacy of international scientific cooperation, and as such has merited not only congratulatory speeches from Messrs Ford and Brezhnev, but also a special commemorative perfume EPAS (Experimental Project Apollo Soyuz, bottles by Revlon, contents by Novaya Zarya) distilled in honour of the occasion. But while the politicians, planners, translators, coordinators and language instructors congratulate themselves on a well-earned triumph, the question remains: what precisely did the joint mission achieve in the scientific field which could not have been accomplished by individual national missions?

Of the "international" experiments carried out by the mission, the artificial solar eclipse made by undocking the two craft, and manoeuvering them into a position in which the Sun was eclipsed by Apollo, and the corona photographed from Soyuz could, theoretically have been effected by two spacecraft of the same "nationality".

The unilateral experiments, *a forteriori*, are likewise independent of the joint nature of the mission. Their juxtaposition in a joint mission does, however, stress a slightly more exotic trend from the American side since in

Sweet smell of success

from Vera Rich

contrast to the fairly routine astrophysical, geographical and biological experiments of the Russians (photography of the zodiacal light, investigation of refraction and transparency of the upper atmosphere, the study of the effect of weightlessness and other conditions of spaceflight on fish embryos and micro-organisms), the American programme included the growing of crystals, the production of lithium fluoride fibres, the casting of magnetic materials, and two electrophoresis experiments (one of West German origin) dealing with the separation of live cells and the isolation of enzymes. It must be remembered, of course, that this Soyuz flight is, from the Soviet point of view, simply a rather more spectacular member of a continuing series, whereas although NASA has two Apollo craft remaining in care and maintenance, there seems no likelihood that with current budgetary restrictions it will be able to launch them.

This, to a certain extent, vitiates the only practical purpose of the joint

mission. In a television interview, a high-ranking Soviet spokesman for the "Interkosmos" council for cooperation in space research stated that according to the International Space Treaty, the contracting parties are bound to implement all possible means to render mutual aid and assistance in the case of emergency. International ventures on perfecting means of docking and rendezvous will therefore continue. Moreover, he said, the future of space research lies in international space stations. The possibility of a Soviet space-craft coming to the aid of a U.S. craft in distress is clearly a tempting propect. The helplessness of the Soviet cosmonauts to effect a salvage operation in the case of the Apollo-13 near-disaster caused keen heart-searching among those who realized what a propaganda boost such a rescue would have been. In all events, unless the U.S. Congress will release the necessary funds, it seems unlikely that Soviet spacecraft will be able to practice the necessary rendezvous and docking manoeuvres, except on each other.

In an ideal future, without budgetary restrictions, where international teams can work together in manned space stations, or even interplanetary flights one outcome of the current

KUBASOV
LEONOV
STAFFORD
SLAYTON

Apollo Soyuz mission will have to be borne in mind—the increased physical stresses observed from the telemetered medical data. The crews were, of course, old for the task, and Slayton had a history of cardiac trouble, but age does not seem the only explanation. A number of factors may well be involved—the intense political significance with which the mission was loaded and the almost embarassingly detailed TV coverage. But these are factors which would with time, gradually fade as international flights became commonplace. It is possible, however, that the strain of operating in a bilingual situation, added to all the other in-flight stresses, may have

proved more wearing than expected. Perhaps therefore the most profitable follow-up of Apollo-Soyuz would be a detailed study of a multinational crew in a simulated long-term mission, with a similar but uninational crew as control experiment, in order to study in depth the additional stresses, if any, caused by international cooperation.

John Gribbin adds: If Apollo-Soyuz was anything more than a political stunt, it was an attempt to pave the way for future joint missions in space. The most glamorous manned mission of the foreseeable future (by the turn of the century) would be a visit to Mars, where the great cost would surely require a global, rather than any national, effort. But before then, and perhaps within five or six years, there is plenty of scope for significant cooperation in Earth orbit.

Curiously, the plans of Soviet and American space scientists dovetail together so well that, politics apart, they seem made for each other. In the best traditions of Tsiolkovski, the USSR is working towards a reasonably large permanent (or semi-permanent) manned space station—but they seem to be intending to build it with the aid

of what are now conventional rockets. On the other hand, the present US effort is towards a superb haulage vehicle for Earth orbit trips, the Shuttle. What could be more natural than to use the Shuttle as a means for supplying the space station of the 1980s?

NASA at least is already well aware of the needs for international collaboration on future projects, and perhaps the most significant single item to be carried by the Shuttle, the Space Laboratory, is being designed and built in Europe, as the European Space Agency's contribution to manned spaceflight. In the heady excitement of last Thursday's golden handshake, the dream of collaboration involving the US, USSR and Europe to fulfill science fiction predictions of permanent orbiting Earth stations semed almost to be a reality; in the more down to Earth days to come, it seems more likely that any such dreams will be blighted by the attitude (on both sides) typified by a remark attributed to one of the US astronauts to the effect that "I like the Russians we've worked with. But that doesn't mean I have to like their lousy system of government." □

A cooperative research programme between the USA and Egypt in the area of remote sensing applications to a national resources survey (in geology, agriculture groundwater and so on) was initiated in 1972 by a proposal submitted by Dr M. Abdel-Hady (an Egyptian scientist who is now Professor at Oklahoma State University, OSU), to the National Science Foundation (NSF) and the Egyptian Academy of Scientific Research.

The project was approved and funded by Oklahoma State University and the NSF for a three-year period ending in 1975, and Professor Abdel-Hady was granted research leave from OSU to direct the effort from Egypt.

The programme so far has covered work in the following areas:

● A geological, structural, drainage and mineral resources survey for the northern region of the Aswan Dam reservoir basin, comprising an area of 68,000 km² on both sides of the river.
● Infrared thermal imaging from aircraft over an extensive area south-west of Cairo. From this investigation, in a typical arid climate, lithological anomalies were significant in demonstrating the role of remote sensing techniques in the discovery of important economic minerals.
● Several studies of the use of remote sensing techniques for the survey and early detection of fungus and nematode diseases in regions of Egypt where important economic crops are grown.

● Geological, water resources, potential oil, mineral resources, and structural maps of the Sinai Peninsula from ERTS-1 Satellite images (under contract with the Ministry of Reconstruction and Housing in Egypt).
● Thermal (infrared), magnetic, radiometric and multispectral photographic aircraft surveys over the entire Suez

---

Remote sensing in Egypt

from Salah Galal, Cairo

Canal Zone, to provile basic surface and subsurface geological maps for a strip 20 km wide, along the entire length of the canal to assist the redevelopment and reconstruction projects in this area and to provide data about the subsurface to the international construction companies in charge of designing the proposed tunnels under the canal.

Several other investigations dealing with agriculture, land reclamation, mineral resources, groundwater and the environment are now proceeding, and more than 10 technical reports and research papers are being prepared from the results of these investigations. These will include studies of the Suez Canal zone (surface and subsurface geological and groundwater survey); the north-west coastal region of Egypt (geological and soil survey, groundwater survey, soil salinity, and agricultural

survey); and the Salheia Project (10,000 km² between the Nile Delta and the Suez Canal). In this project a regional and detailed geological, soil hydrological and crop survey is being carried out, with the aim of reclaiming 10.000 acres of land.

Other projects now being negotiated include a crop survey inventory and early detection of some nematode and fungus diseases in major economic crops in Egypt; a survey of iron ore deposits over large areas of the Western Desert; a regional geological soil and water resources survey for 100.000 km² west of Aswan; and a regional geological, structural and petroleum and mineral resources survey of a large area in the Western and Eastern Deserts of Egypt.

Regional groundwater investigations and mapping of groundwater reservoirs are planned for the Nubian Sandstone in Egypt, northern Sudan and Libya, and regional and general area reconnaissance will take place in the El-Sudd region in Sudan for the planning and location of the Jongoli Canalisation Project (an important water resources and water conservation project planned jointly by the governments of Egypt and Sudan). Cooperation in carrying out this investigation by satellite image interpretation and by means of aircraft is now being negotiated with representatives of Egypt and Sudan at the Higher Commission for the Upper Nile Waters, Ministry of Irrigation.

Brazil and Germany do a nuclear deal

from Ian Bridges, Bonn

IN Bonn at the end of June foreign ministers Hans-Dietrich Genscher of the Federal German Republic and Antonio Azeredó da Silveira put their signatures to an epoch-making treaty. Under its terms the two countries are to develop, using German know-how and Brazilian capital and uranium, a complete nuclear fuel cycle, from mining to waste disposal. This was the German's first breakthrough in the complex game of world-scale technological and commercial diplomacy. The range of reactions to the deal is a reflection of the many and diverse planes on which it will have an impact. Within Germany, the accent was put on the value of the commercial contracts and their spin-off in balance of payments terms. In Brazil, relief at ensuring energy supplies for the future, when hydroelectric power will have been stretched to its limits, mingles with satisfaction at an assertion of independence from Uncle Sam. In the United States, Congress echoed to expressions of high moral indignation that Germany, of all countries, should have given the Brazilians the wherewithal to "make the bomb"

Shrewder observers noted that the deal had long-term political and strategic implications, relieving the Federal Republic of dependence on US enriched uranium in the closing decades of the century. And in the background, ecologists lamented that it opened the way to a further spread of nuclear power, with potentially disastrous implications for the future of mankind, whilst scientists speculated about this as a test case for the effectiveness of control over the peaceful use of a new enrichment method.

Technically, the nuclear complex that is to be built at Angra dos Rios, on the Itaorna Bay 130 miles of Rio de Janeiro, represents a double first. It is the first complete nuclear power cycle to have been sold. This indeed was the key selling point with the Brazilian government, who could not obtain a similar offer from Westinghouse, whose single 600 MW standard plant is currently being built within a few hundred yards of the site for the new complex.

By the time the cycle is completed (within 10 years), it will cover prospecting for and mining of uranium within Brazil, which is known to have such resources; a uranium enrichment plant; two completed 1,300 MW pressurised water reactors, with another six reactors on the way; a pilot plant for making reactor fuel elements, with a commercial plant to follow; and a

plant for processing used fuel. But in this list, one thing stands out—the enrichment plant. For this is the first commercial sale of the method known as nozzle or vertical wall centrifuge (German: *Trenndusen*). This has been developed by the Federal German nuclear research centre in Karlsruhe— and in parallel by the South Africans —but has not been used commercially so far.

It was mainly the ability to sell an enrichment process—plus their proven engineering ability across the whole field—which gave the Germans the edge over the Americans Brazil's economic planners had begun to find themselves doubly squeezed on the energy front With maximum exploitation of the country's considerable hydroelectric power resources already foreseeable, they had made the switch to oil when the world oil crisis hit them. For a country which had just begun to master about the worst runaway inflation rate in the world, this was a shock. But thinking in terms of nuclear power as a medium-term alternative, they ran into the hard fact of the predicted enriched uranium shortage, already making itself felt, and due to be worse at the end of the 1980s. So the nuclear choice looked like restoring a dependence on the US which they were trying to break.

The answer was to develop a full fuel cycle. enriching their own uranium. But the gaseous diffusion method, the only one the US companies could theoretically have provided, could not be made available on non-proliferation grounds, because it can be used to enrich up to the levels needed for military uses. The Anglo-German–Dutch centrifuge method is also subject to the tightest restrictions, under the secret clauses of a deal with the United States, and is in any case not yet available commercially. The German method, on which the scientists in Karlsruhe have concentrated, is generally recognised as being "proliferation safe"—meaning that it can only effectively be used to enrich up to the low levels, 3 5% or so, needed for peaceful uses. It seems that any adaptation of the vast installations to achieve higher levels, would be so easily controlled.
costly and so conspicuous as to be

In these circumstances, the wave of protest in the US Congress against the German–Brazilian deal, specifically on the grounds that it meant "giving the Brazilians the bomb", seems hard to explain, unless resulting from inadequate technical knowledge. Although Brazil is not a signatory of the non-proliferation treaty, the Germans made it a condition of the sale that Brazil should accept controls by the International Atomic Energy Agency

in Vienna. The powerful pressure which the US government also tried to put on the Federal Republic, against the deal, seemed to be more general. But as the Germans tended to point out, if the danger foreseen was that plutonium from the reactors, after their first period of operation, was seen as giving Brazil a military potential, this is a danger equally present in US sales of reactors to countries at least as potentially dangerous as Brazil, such as Iran or Egypt.

One expert has suggested a third possibility: one of the inventors of the centrifuge spent many years after the War, when working on the project, in Brazil before returning to Germany, and it would thus be surprising, said this source, if the Brazilians had not discreetly gone on with the centrifuge; but this was queried by others who pointed out that the bottleneck in centrifuge development was not the invention itself but the difficult high-temperature technology of the bearings, something the Brazilians are not equipped to master. In short, if Brazil is suspect as a potential maker of bombs, the breakthrough is going to come anyway As for innuendoes that the deal also gives the Germans the potential of developing a bomb together with the Brazilians, there seems no foundation for them—at least as long as the Federal Republic retains its present political balance.

From the Federal German point of view, in any case, there is no doubt that the Brazilian deal was pushed through essentially for its commercial value. For the Germans it was a lucky break when they were beginning to despair of ending the Americans' domination of the world market for nuclear technology and engineering. An estimated 15,000 million DM of public money has been poured into the nuclear industry, which is now geared up to produce and sell reactors commercially. Kraftwerk Union AG, joint subsidiary of Siemens and AEG (though AEG are currently selling out) can produce and sell six reactors a year Given a six-year construction cycle, this means a potential of 36 contracts running in parallel. Currently, there are orders for only 17. Except in neighbouring countries—Austria, Switzerland, Holland—KWU has almost lost out to Westinghouse or General Electric—not least because of the all-out diplomatic back-up enjoyed by the Americans. Thus the Germans recently won an order from the Shah of Persia for two reactors—only to see the Americans carry off a contract shortly afterwards for a programme of eight over a 10-year period.

In Brazil, precisely the reverse has happened. The starting-point was a straight bi-lateral inter-governmental

pact, signed in 1969, on the exchange of scientific information. From the start the Brazilians showed the most interest in the nuclear sector, and soon talked about a full fuel cycle, but were not taken seriously. In 1972 the Germans made a bid for the reactors the Brazilians had decided on, but when they saw Westinghouse carry off the first contract they almost gave up. Then, in the spring of 1974, came a request for the full fuel cycle, this time serious. The talks, on which German industry was brought in, were kept secret for a long time.

When it did come out, in the early summer, the news was an enormous fillip for a German economy reeling under bad news. For years, the prosperity of the Federal Republic had been export-led, with booming sales of investment goods abroad balancing out the staid performance of private consumption within the country. In 1975 exports had begun to flag, reflecting the recessions elsewhere in the industrialised world. Of the investment in the nuclear programme planned with the Brazilians, an estimated 12,000 million DM was to be spent in the Federal Republic over 15 years. There was guaranteed work for the 13,000 strong skilled workforce at KWU, plus sub-contracting to 300 or more firms for 80% of the material to be used. But there can be no doubt that nuclear development proper will spearhead a wider commercial breakthrough. There will clearly be a need for steel produced on the spot, and for a range of engineering firms; and around this tremendous source of electric power, fertiliser and chemical plant will proliferate.

Far more than was generally realised at first, the Federal Republic and Brazil have gone into partnership in the business not just of producing nuclear power, but of supplying the kits for others to do the same. The briefest summary of the full range of cooperation arrangements illustrates this.

● a joint company has been set up to carry out prospecting and mining of uranium. A Brazilian state company, Nuclebras, has 51% and Germany's Uran Gesellschaft the other 49%.

● the same Nuclebras will take part in developing the vertical wall enrichment plant, which will be built jointly in Brazil by Steag and Interatom.

● KWU and the specialised company RBU are to build first a pilot plant and then a commercial one for making nuclear fuel elements for Brazilian reactors; and significantly Nuclebras will have 70% and KWU 30% in a separate company set up to market fuel elements.

● for re-treatment of nuclear fuel, Nuclebras will have two German firms

THAT much-publicised proposal to reestablish a science policy office in the White House, announced by President Ford in May, is making glacial progress through Congress, and it now seems certain that the new arrangement will not be in place until late autumn. That would be too late for the office to play much part in deliberations on the 1977 budget and, with the Presidential elections by then in full swing, it may be difficult to persuade a top flight person to head the office for what could be a short stint.

The House Committee on Science and Technology, which is dealing with the proposal in the House, completed hearings on the measure last month, and it is not likely to take any formal action until after Congress returns from its August recess. In the Senate, meanwhile, the proposal has been referred to three different committees, and joint hearings are being planned for September. Congressional staff members concerned with the legislation suggest that it will be at least October before a bill is passed and sent to President Ford.

Ford's proposal, which was formally spelled out in a bill sent to Congress early in June, would establish a small office consisting of about 15 people—a number which was "pulled out of the air", according to testimony given by Vice-president Rockefeller—and a budget of about $1–1.5 million a year. Although it would be concerned chiefly with helping to formulate Administration policy on non-military science and technology, the office would play a rôle in military matters "when asked".

Congress is likely to give Ford

pretty much what he asked for, although it is likely to stipulate that the head of the office, who will bear the title of Science Adviser to the President, should be approved by the Senate before he is officially appointed. It is also possible that Congress will spell out the office's areas of responsibility in some detail, particularly in regard to its rôle in military science policy making.

During the House Science and Technology Committee's hearings, the proposal was warmly endorsed by several prominent scientists who had long been protesting former President Nixon's decision, in January, 1973, to scrap the White House science policy arrangements established by President Eisenhower. Having persuaded President Ford to reverse his predecessor's decision, however, the proposal's supporters have won only half the battle—the other half is to ensure that the arrangement is used effectively when it is in place, and that may be more difficult.

● Echoing warnings issued in a recent report of a committee of the National Academy of Sciences, the Geological Survey, which is part of the Department of Interior, pointed out last week that the United States is becoming increasingly dependent on imports of several important minerals. The survey urged that a national policy aimed at conserving scarce minerals and substituting others in some uses, should be adopted. By the year 2000, the Geological Survey predicts that the United States will be completely dependent on imports for 12 commodities, more than 75% dependent on imports for another 19, and more than 50% dependent on imports for a further 26 commodities.

acting as partners: KEWA and UHDE.

● only the first two reactors of the eight planned are to be delivered on a turnkey basis by KWU, with work starting on one in 1976 and the other in 1978. Nuclebras and KWU are going 75–25 in a nuclear engineering company, while the Austrian firm Voest takes part in a nuclear components company.

If there is thus a vast commercial pay-off for the Federal Republic, there is also a longer-term advantage of capital importance. The Federal Republic will have guaranteed access to uranium—and a guaranteed source of enriched uranium just at the time the squeeze starts operating. This means escaping from the present dilemma of having to rely on either the USA or the Soviet Union to provide the enriched uranium. The uncertainties of this were underlined earlier this year

when supplies to the European Community were halted without warning or consultation, formally because of inadequate security. US pressures on economically sensitive areas like this are ill-received even by such loyal "Atlantic" partners as chancellor Helmut Schmidt—and the calls on the Federal Republic to drop the Brazilian deal tended to confirm the suspicions of those who had opposed German signature of the Non-Proliferation Treaty on the grounds that it would be used by US interests to hinder the Germans on world markets.

Thus the German-Brazilian deal is another step towards a changed pattern of world influence in terms of economic, commercial and technological weight and independence. The strongest of the western European industrial countries has linked hands with the country which is potentially a "first-league" member. □

Plant history in the Soviet Union

from Vera Rich

THE meeting of the Twelfth International Botanical Congress, which opened in Leningrad on July 6, has yielded a number of interesting reports from Russian botanists. In the field of palaeobotany, Leningrad palaeobotanist Boris Timofeev reported finding fossils of unicellular plant life in a 6-km core sample taken from the Ukrainian shield. This find, he claims, should be dated at 2,000 million years BP. It was also stated that this discovery will be important in the dating of strata of ancient rock, and that it will be of particular importance in oil and gas prospecting—though this claim seems somewhat obscure, since fuel deposits are conventionally attributed to the Carboniferous and not the Palaeozoic Period.

Also in the field of dating, a team of biologists and archaeologists have developed a "unified dendrochronological scale" for estimating climatic changes in the last 1,200 years More than 8,000 specimens of wood (mostly pine and fir) were used, from the surviving remnants of bulwarks, fortresses, log-built palaces, water conduits and the wooden pavements which were a notable feature of a number of major cities of the state of ancient Rus', notably Novgorod. A reliable time scale for eastern Slavonic history begins only in 988 AD with the conversion to Christianity of the Principality of Kiev, and the exact claim that the most ancient logs used (from sites at Pskov) date back to 770 AD shows rather too great an apparent precision. Equally puzzling is the claim that the data collected not only refer the growth of trees to the general picture of climatic change, but also to "events of cosmic origin". In particular, retarded tree growth is said to be correlated with chronological data on the flares of supernovae, intense meteorite showers and "dimness" of the Sun. This, however, raises the problem of how the original dating is arrived at Although presumably certain wood specimens can be clearly attributed to the dateable destruction of a given city (and in at least one case, the sack of Novgorod by Vseslav "the Wizard" in 1066, there is an exact coincidence with a cosmological phenomenon—the appearance of Halley's comet), the question how long the wood had been in use when destroyed remains open. If the dating involved is simply carbon-14 measurements, then the margin of error is surely too great to effect a definite correlation to a definite historical event. One suspects that, at some point, some circular argument has entered into the discussion.

Meanwhile, in the field of modern botany, an engineering team reports the development and use of "climatrons"—artificial climate machines with special light bulbs to stimulate different spectra of solar radiation, and special humidity and heat control systems, which provide a short cut to the evolution of new varieties of cultivated plants.

One such new variety (whether evolved in a climatron or not is unclear) is the splendid grass reported by Alexsandr Fedorov, which is intended as the cattle feed of the future and which grows to a height of some 5 m. In his message of greetings to the conference, Mr Kosygin, Chairman of the Council of Ministers of the USSR, expressed his view that on botany, "to a considerable degree, are based achievements in many branches of practical human activity, including agriculture " Certainly, at first glance, the 5 m grass must rank as such an achievement. Presumably it is intended for silage, rather than direct grazing Even so, the practical problems involved in its production on a commercial scale may well prove somewhat of an embarrassment to stock rearers, haymakers and the manufacturers of agricultural machinery.

● THE grainlands of the Steppe have been a potential cause of political tension throughout their history—indeed, there are those who would attribute the underlying cause of the Trojan war to the need of the Greek tribes to break through the Trojan blockade of the Dardanelles to the wheatlands north of the Euxine The nineteenth century brought, in Britain, the vexed issues of the corn laws and free trade, both of which assumed an inexhaustible source of wheat in the then Russian empire. In the twentieth century, Hitler envisaged an annexed Ukraine as the breadbasket of his Thousand-Year Reich, relying, presumably on the traditional picture of its fertility, rather than on the events of recent history For, under Soviet rule, grain production has consistently failed to reach the

planned target figures, and, in spite of the great drive to open up the virgin lands of Siberia to the plough, Soviet agriculture is no longer able to satisfy its home market, and this year, once again, must buy some 10 million tons of grain from abroad.

According to the Soviet press, most of the blame is to be placed on administrative difficulties—farmers so busy with paperwork on crop returns that they have no time to go about their primary business of cultivating and harvesting, machines standing idle for lack of spare parts or maintenance, or the occasional "wrecker" (a recent issue of *Pravda* reports that collective farmers not infrequently feed loaves of intended for human consumption to the livestock which they are allowed to tend as their personal property in their spare time). This is, however, a relatively easy solution to offer. The real situation, however, is more complex, and involves not only misapplication of the administrative process, but the whole Soviet system of planned agriculture.

In spite of the division of the Soviet Union into Union and Autonomous Republics, and (on a somewhat different basis) into economic regions, all planning in agriculture, as in all other types of production, originates in Moscow, and, it would seem, is often carried out with little regard for local conditions Stalin once, in speaking to Churchill about the "bad and difficult" time of collectivisation claimed as one of its advantages: "Not only have we vastly increased the food supply, but we have improved the quality of the grain beyond measure. All kinds of grain used to be grown. Now no one is allowed to sow any sort but the standard Soviet grain from one end of our country to the other."

Such a statement, of course, assumes that the "standard Soviet grain" is equally suitable for all the highly diverse habitats of one-sixth of the land surface of the Earth. In fact, a number of standard grain types have been developed for different conditions, and the claims made for them are impressive. A *Novosti* press release issued this year mentions eight valuable varieties of wheat developed at the Institute of Wheat Breeding and Seed Growing at Mironovka, south of Kiev, including, not only winter wheat, but an "insurance" type spring wheat "Mironovskaya-808" which can be used to supplement the grain balance when the winter wheat crop is damaged or frozen. This however raises the question, if winter wheat is at risk in a given area, why not concentrate on spring wheat altogether?

During the "maize craze" of the 1950s this crop was sown indiscriminately throughout the Soviet Union,

including areas such as Byelorussia where it could not be expected to ripen (Khrushchev suggested that in such areas unripe maize could be used as fodder). Even in more southern areas, the crop was introduced without proper arrangements being made for its harvesting, and on one occasion, when Khrushchev was to make a winter tour of Ukraine, the local organisers had no alternative but to flatten with large rollers the ungathered maize cobs that were protruding through the snow!

The ecological problems involved in such centralised planning may also be not fully understood The forestry conservation measures introduced in the Carpathians last May were intended not only to conserve the diminishing timber resources of the area, but also to restore the water-table of the agricultural areas of western Ukraine. The horizon-to-horizon culti-

vation of the virgin lands, without wind-breaks or chequering of fields, has led to considerable erosion.

Another problem is that of crop-rotation At the Twenty-Fourth Party Congress in 1971, the Minister of Agriculture, Vladimir Matskevich, stated that "correct" crop rotation had now been introduced on about 87% of arable land, but no details are available on the patterns of rotation involved. Unofficial reports, however, indicate a general tendency away from traditional patterns, and although the use of leguminous plants for nitrogen fixation is highly esteemed, it is chemical fertilisers that are seen as the basis of Soviet agriculture. In his report to the Twenty-Fourth Congress, Matskevich spoke of "mechanization, electrification and chemicalisation" as the basis for the intensification of agriculture, and "popular" articles appear in

the Soviet press on the advantages of chemical fertilisers.

From time to time, the Soviet press reports the triumph of small agricultural teams, working in virtually small-holding conditions, who have been able to increase the output of the land at their disposal by impressive percentages This can, of course, only be an experimental scheme—its large-scale implementation would undermine the basic theory of Soviet agriculture, collectivisation

● In the article *Soviet meetings, great and small* (*Nature*, **256**, July 17, 1975, page 160), paragraph 6 should have begun "The case of Dr Gluzman, whose incarceration in a prison camp seems to have been the direct result of his preparation of a report on psychiatric malpractice in the case of General Grigorenko, was raised at last year's Annual Meeting." □

IN these days of world-wide food shortages and rising food prices, it is hard to believe that a nation whose prairie provinces are often thought of as the breadbasket of the world neglects its agricultural and veterinary medicine schools. Yet that, according to the deans of Canada's 11 faculties of agriculture and veterinary medicine, is exactly what is happening

Through the Science Council of Canada, they recently published a statement saying the faculties "find themselves chronically underfinanced despite widespread government and public lip-service to the essential importance of food production and the need to apply education and research to its increase."

The "national statement" by the 11 deans went on to say the schools "lack funds for current activities other than teaching students; they do not have enough staff to achieve appropriately small class-sizes; and they have neither the time nor the money to undertake research that is needed and of which they are otherwise capable."

As a result, the deans declared, the faculties are intellectual resources that are being exploited to only a fraction of their potential.

It is not the first time the problems of science in agriculture in Canada have been made public Two previous Science Council publications identified a lack of coordination in research, particularly between the federal government and the universities (*Agricultural Science in Canada*, Background Study No 10, 1970; and *Two Blades of Grass*, Report No 12, 1971, Information Canada) Roger Gaudry, recently-retired chairman of the Council, also drew attention to the problems in his Annual Report for 1972–73.

Nor does the neglect of the agriculture and veterinary faculties mean that agricultural research *per se* is neglected in Canada. On the contrary, in 1972–73, Agriculture Canada (the federal government department) spent $90 million on research and development alone. But by contrast, the 11 agriculture and veterinary medicine faculties received total funding in that year of less than $50 million—only

---

Agricultural research at risk

from David Spurgeon, Ottawa

$15.5 million of which was for research.

And despite the inauguration in 1972 by the Ministry of State for Science and Technology of a "make-or-buy" policy—under which federal government departments were directed to contract out research where possible —Agriculture Canada remains the only department that spends about 99 per cent of its research budget in-house

The neglect of agricultural science lies chiefly in the small proportion of total R & D funds spent on agricultural research, and the lack of national planning, in addition to the over-emphasis on in-house governmental research.

"Out of about $1 5 billion a year spent on scientific and technological research in Canada, agriculture and its related sciences get much less than 10 per cent," says the dean's statement. "For want of staff or funds, needed research projects that promise high 'payoff' simply do not get started "

What makes the situation worse, they continued, is that what agricultural and food research is done is neither planned nor evaluated on a national scale in terms of its need and productivity.

Canada's three veterinary colleges have to turn away highly-qualified applicants for lack of space, the deans said The four Atlantic provinces together are rarely permitted to place more than six students a year at the only English-speaking institution in eastern Canada, the Ontario Veterinary College.

"The shortage of livestock veterinarians is a national problem for which every Canadian consumer is ultimately paying in increased meat prices."

In certain agricultural disciplines, professionals are also in short supply, to such an extent that government agencies have had to hire a significant proportion of their professional staffs outside Canada.

The deans' report was addressed primarily to the federal government, the provincial governments, and the food industries, according to Dr. Gaudry, who wrote the introduction But it obviously is hoped many ordinary Canadians will read it as well

It constitutes a direct challenge to the governments· "We can choose programmed ignorance and increasing risk in our food-production system, resulting from science policy by accident and a failure to understand the relationship between teaching, research and public service in the universities Or we can choose to develop a fully-articulated set of national agricultural policies and support strategies, in which each sector's distinctive advantages are used efficiently."

news and views

Microtubules and membrane topography

from Robert Shields

THE fluid-mosaic model of the cell membrane is rapidly attaining the status of the central dogma of membrane biology. Acording to this model (Singer and Nicholson, *Science*, **175**, 720; 1972) proteins are free to diffuse in the plane of the lipid membrane and assume random or homogeneous distributions over the cell surface.

More recently, evidence has been produced that suggests submembranous structures such as microtubules and microfilaments may also help to modulate the cell surface molecules.

When mouse B lymphocytes, which have immunoglobulin (Ig) molecules on their surfaces, are stained with fluorescent antibody to mouse immunoglobulin (anti-Ig), several patterns of fluorescence may be observed depending on the experimental conditions. If monovalent anti-Ig is used the cells are stained diffusely, suggesting a random distribution of Ig molecules over the cell. At low temperatures with divalent antibodies a patchy pattern of stain is seen implying partial aggregation of Ig molecules. When the temperature is raised the patches coalesce to form a fluorescent cap at one pole of the cell Patch and cap formation are not unique to lymphoid cells and quite a wide variety of cells including mouse fibroblasts have been successfully capped using anti-H₂ antibody (Edidin and Weiss, *Proc. natn. Acad. Sci U.S A.*, **69**, 2456; 1972).

The formation of patches is rather similar to the classical antigen–antibody reaction in solution in that it requires the antibody to be divalent and antigen to be present in multiple sites But the formation of caps is more complex than formation of patches, in that it is dependent on cellular metabolism, especially ATP synthesis. Patch formation is passive and depends on bivalent anti-Ig molecules collecting Igs by diffusion but several lines of evidence suggest that microtubules and microfilaments may play a part in the translocation of patches to form caps.

Perhaps the strongest evidence for the involvement of microtubules in the movement of cell surface proteins comes from work with the plant lectin concanavalin A (Con A) and B lymphocytes Native Con A is multivalent and

reacts with the α methyl-D-mannoside residues that form part of many of the glycoproteins of the cell surface. Con A acts as a kind of honorary antibody and at low doses will lead to capping of its receptors on B lymphocytes At high concentrations of Con A, however, no patching or capping is observed (Yahara and Edelman, *Proc. natn Acad Sci. U.S.A.*, **69**, 608; 1972). High doses of Con A inhibit not only the capping of Con A receptors but also the capping of Ig molecules with anti-Ig. If the cells are treated with colchicine or the temperature lowered to 4 °C, the cells washed free of excess Con A and the temperature shifted to 37 °C, caps will form.

An attractive explanation of these observations was provided by Yahara and Edelman (*Expl Cell Res.*, **91**, 125; 1975). They believe that molecules on the cell surface (such as Ig or Con A receptors) may exist in two interconvertible states, a free state where they are able to diffuse laterally in the lipid matrix, and a bound state where they interact directly or indirectly with microtubular structures inside the cell. Interaction of Con A with its receptors would lead to the binding of free receptors to a lattice of microtubules. The lattice, which is caused to form by high doses of Con A, also interacts with Ig molecules and prevents their diffusion to form a cap. When colchicine or other microtubule-disrupting agents are added, or the temperature is lowered (which also breaks up microtubules) the lattice would break up, allowing free diffusion of surface molecules and the formation of caps. But before this model for receptor modulation can be accepted other possible explanations of the results should be considered.

One possibility is that the binding of Con A to the lymphocytes induces a phase transition of the membrane and decreases the membrane fluidity This would inhibit the free translocation of Ig and other molecules in the lipid phase. Colchicine and vinblastin at the high doses used by Edelman could conceivably interact directly with the cell membrane increasing the membrane fluidity and permitting receptor movement. Lowering the temperature

might lead to phasing out of the cell membrane into fluid and non-fluid domains and so permit increased receptor movement (Petit and Edidin, *Science*, **184**, 1183; 1974).

These possibilities could be tested quite simply by using fluorescent lipid probes inserted into the cell membrane to measure membrane fluidity by fluorescent polarisation before and after Con A or colcemid treatment Although the evidence is rather sketchy it seems that binding of lectins to cells is more likely to increase than to decrease membrane fluidity (Toyoshima and Osawa, *J. biol Chem*, **250**, 1655; 1975) Furthermore lumi-colchicine, an isomer of colchicine which reacts equally well with plasma membranes (Stadler and Franks, *J. Cell. Biol.*, **60**, 297; 1974) but not with microtubules, fails to relieve the Con A-induced inhibition of Ig capping (Yahara and Edelman, *Nature*, **236**, 152; 1973). These experiments would seem to indicate that microtubules are involved in receptor movement.

A further possibility is that the binding of Con A to cells leads to the formation of aggregates on the cell surface that trap Ig molecules and so prevent their diffusion. Since there are about 1 4×10⁶ Con A receptors and about one thirtieth as many Ig molecules on the cell surface this is a possibility. To counter it, Edelman calculated that even at saturating doses of Con A, Con A receptor complexes occupy only 1 % of the cell surface and so are unlikely to interfere directly with the free diffusion of Ig molecules (Edelman *et al*, *Proc. natn. Acad. Sci. U.S A.*, **70**, 1442; 1973).

A related and more plausible possibility is that, since Con A will interact with many different glycoproteins through α methyl-D-mannoside residues, Con A may in fact directly crosslink Ig molecules at the cell surface. rather than through some submembranous microtubular structure This idea was given credence by the observation that Con A was able to cap Ig molecules without the need for anti-Ig Furthermore this co-capping takes place even in the presence of colchicine or at 4 °C, conditions that should disrupt microtubular structures. Con A also

induced co-capping of other molecules, such as the H-2 and Θ antigens in splenic lymphocytes, without recourse to use of the specific antibodies. In the light of these results it was vital to show that the Con A-induced inhibition of Ig motility was not an uninteresting artefact due to crosslinking at the cell surface but that microtubular structures were involved.

To do this Yahara and Edelman took advantage of the relative difference in size between the B lymphocyte and blood platelets (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 1579; 1975). They conjugated the small platelets with Con A and studied their interaction with splenic lymphocytes. The much larger lymphocytes could bind several of the platelets through the conjugated Con A, and the platelet distribution was random over the lymphocyte surface. The idea was to complex only a small proportion of the total Con A receptors of the lymphocytes and to see if Ig movement was still restricted. Staining the platelet-treated lymphocytes with fluorescent Con A revealed that only a fraction of the lymphocyte Con A receptors were bound to the platelets; furthermore the Con A receptors not bound by the platelets were distributed diffusely over the lymphocyte surface. The binding of more than ten platelets per cell was sufficient to prevent anti-Ig-induced capping of Ig molecules, showing that Ig redistribution was blocked by the occupation of only a fraction of the Con A receptors. When the lymphocytes with their bound platelets were treated with colchicine they showed polar or cap-like distribution of the platelets on their surfaces. But when these colchicine-treated cells were examined with fluorescent anti-Ig for the distribution of Ig molecules, no co-capping of Ig molecules with the Con A platelets was seen· the motion of bound Con A receptors and Ig molecules was now independent. This provides the strongest evidence that Con A receptor modulation of Ig on the surface of B lymphocytes involves microtubular structures and is not an artefact generated by crosslinking of Ig molecules to Con A receptors on the cell surface.

The evidence for the involvement of microfilaments in the movement of molecules in the cell membrane is even more indirect and depends on the use of the drug cytochalasin B This agent has had a rather poor press recently and it is not clear how it interferes with microfilament function. A further complication is that different preparations of this drug have different activities. What is fairly clear however is that cytochalasin B will interfere with cap formation and in the right condi-

tions will lead to an 80% inhibition of cap formation induced by anti-Ig. When used in conjunction with colchicine, cap formation is almost completely blocked. The addition of cytochalasin B to cells that have already capped leads to a dissolution of the capped molecules (de Petris, *Nature*, **250**, 54; 1974)

These findings suggest that microfilaments as well as microtubules have a role in the movement of molecules on the cell surface. What is required now is some more direct evidence of the linkage of molecules on the cell surface to microtubules and microfilaments in the cytoplasm.

Another overexploited fishery

from our
Marine Vertebrate Correspondent

OVEREXPLOITATION of fishery resources is no new phenomenon. Examples range in emotive potency from the state of whale stocks, through the decline in numbers of the Atlantic salmon, to the humble North Sea herring and the Californian sardine. Developments in fishery technology, aimed at improving the catch–effort ratio, have generally produced increasingly efficient means of exhausting the stock.

There is no worse offender in this respect than the Japanese high-seas fishery for tuna which during the last two decades exhausted the stocks in the southern Indian Ocean and the western Pacific, before transferring its operations to the Atlantic and the eastern Pacific Oceans. The consequences of this fishery on the billfishes (sailfish and marlins) of the eastern Pacific are discussed in a recent paper by Talbot and Wares (*Trans. Am. Fisheries Soc.*, **104** (1), 1–12; 1975).

By 1963 the Japanese fishery had expanded to 10° north latitude, offshore of Central America, and heavy catches of yellow-fin tuna (*Thunnus albacares*) and striped marlin (*Tetrapturus audax*) were made. In time it spread further north and the marlin became the major exploited species. The success of this fishery resulted in increased fishing effort and in 1964 the catch of this one species approached 300,000 fish. Thereafter the catch declined until 1967, reached a high point in 1968 of around 320,000 fish, but dropped dramatically the next year to just over 200,000. A comparable change has been observed in the catches of blue marlin (*Makaira nigricans*) which attained a maximum (nearly 80,000) in 1963 and thereafter declined severely, only increasing slightly (to 30,000) in 1969. The sailfish (*Istiophorus platypterus*) similarly shows an overall decline

from a peak of nearly 400,000 fish in 1965 to 100,000 in 1969.

Talbot and Wares examine the possibility that these changes in catch may reflect abundance variations caused by differing sea temperatures but, except for one area, they find little evidence to support this. They do, however, find clear evidence of overfishing. The first peaks of abundance for each of the billfish species can be related to increased fishing effort, and the decline in catch has persisted in some cases even though the number of hooks fished has increased. Expressed in terms of hook rate (the catch per hundred hooks), it seems that the striped marlin declined marginally from 0.7 to 0 3 between 1968 and 1969, the blue marlin catch fell from 0.35 to 0.05 between 1959 and 1969, while the sailfish catch fell from nearly 3 to 1.2 between 1965 and 1969. Clearly, in the cases of both the blue marlin and the sailfish the decline has been severe and indicates overfishing and depletion of the stocks. And the average weight of these fish has declined, which is also evidence of overfishing.

The reduction in numbers and weight of these billfishes may have considerable consequences for the sport fishery of the western Mexican and Californian coasts This sport fishery has provided an important contribution to the income of Pacific Mexico since the second world war. Talbot and Wares point out that the reduction in average size of the fish caught has already made it a less spectacular sport But they suggest that with the declining returns per unit effort the Japanese fishery may well decrease, leaving enough of the smaller billfishes to maintain the sport fishery

Exciting days over for reverse transcriptase?

from Karin Moelling

IT is five years now since Temin and Baltimore reported the existence in RNA tumour viruses of a reverse transcriptase which can make a DNA copy from the RNA genome. For some time progress on the characterisation of this new type of enzyme filled the front pages of the journals. But results on the characterisation of the enzyme are no longer so spectacular, and have become matters for hard working biochemists.

What do we know about the viral reverse transcriptase? Research was concentrated on the avian viral reverse transcriptase as large amounts of avian viruses can be isolated — and were generously supplied by J. Beard.

We learnt that the purified enzyme transcribes natural viral RNA very efficiently, for which it requires an RNA primer which was identified as a tryptophan tRNA (Dahlberg *et al*, *J. Virol.* **13**, 1126, 1974). In addition to the RNA-dependent DNA polymerase a DNA-dependent DNA polymerase is involved in the synthesis of the double-stranded DNA product Furthermore an RNase H activity seems to play a part. All these activities are coded for by the viral genome as was shown with avian viral temperature sensitive (ts) mutants (Verma, Mason, Drost and Baltimore, *Nature*, **251**, 27; 1974).

Enzyme from murine virus

One would have expected it to be simply a matter of routine to generalise the knowledge about avian viral reverse transcriptases to those of viruses from other species. This was, however, not the case. Mammalian viral reverse transcriptases could copy synthetic templates such as poly (rA). oligo (dT)—but did not seem to be able to carry out what they were given their name for doing, the reverse transcription of exogenous viral RNA into DNA! The RNase H seemed to be absent as well.

So the initially fast progress was followed by a series of negative results. But the results obtained with the avian enzyme were so well established that nobody could have seriously questioned their relevance also for mammalian viral enzymes. Therefore it did not come as a surprise when finally three detailed analyses showed that murine leukaemia viral reverse transcriptases had all the properties of avian enzymes: natural RNA transcription, presence of RNase H in a single polypeptide with the polymerase activities (in two of the three reports) and proof of its viral, not cellular, origin on the basis of its exonucleolytic mode of action (Moelling, *Virology*, **62**, 49; 1974; Gerard and Grandgenett, *J. Virol.*, **15**, 785; 1975; Verma, *J. Virol.*, **15**, 843; 1975).

What was the problem with the mammalian viruses? Apart from inadequate assay conditions used in some cases the starting material seemed to be important Mammalian viruses may be more sensitive to manipulation and the enzyme seems to be inactivated more readily.

As far as the RNase H is concerned, there was still a confusing matter left to be settled· Gallo's group reported that they could separate the RNase H from the viral reverse transcriptase (Wu, Sarngadharan, and Gallo, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1871; 1974) which suggested that it could be a host cell contaminant.

This problem seems to have been nicely clarified by the latest paper of Gerard and Grandgenett (*J. Virol.*, **15**, 785; 1975). Like good diplomats they show that both results are correct: there is one RNase H which can be removed from the transcriptase; another (the viral one), which cannot, is present in smaller amounts and may have been missed by Gallo's group. The one which can be removed is very small and looks to me on first sight similar to the 'mini'-RNase H recently described as one of the cellular RNase H molecules in calf thymus cells (Buesen and Hausen, *Eur. J. Biochem.*, **52**, 179; 1975). It remains to be shown that the removable RNase H is really of cellular origin. The ultimate proof of the presence of viral RNase H in the single polypeptide murine viral reverse transcriptase molecule must come, as with the avian enzyme, from viral ts mutants.

The enzymatic properties of the reverse transcriptases of viruses from avian and at least one mammalian species, the murine one, seem quite similar. But in spite of their enzymatic relatedness they look structurally quite different: the avian reverse transcriptase has been described as a two polypeptide complex of molecular weights 110,000 and 70,000, called β and α respectively, and the murine viral reverse transcriptase consists of a single one. But there are also reports (Verma *et al.*, *J. Virol*, **13**, 1075; 1974; Gerard and Grandgenett, *J. Virol.*, **15**, 785; 1975) which offer two or even three subunits. The number of subunits may not be very relevant — except if some of them are host factors which supply functions missing in the single viral subunit. Indeed the purified viral enzymes are deficient in properties which they must exhibit *in vivo*: *in vitro* they are only capable of synthesising short DNA sequences which are not representative of the total genome.

I do not believe, however, that some of the described subunits are cellular factors. It seems much more likely that the smaller ones are degradation products of a larger one. So far, this has only been shown in the case of the avian viral reverse transcriptase whose larger polypeptide spontaneously undergoes transition to the smaller one, a process which can be stimulated by mild protease treatment (Moelling, *Cold Spring Harb. Symp. quant. Biol.* **39**, 969; 1974). Tryptic peptide pattern analysis by Gibson and Verma nicely confirms this relationship between β and α (*Proc. natn. Acad. Sci. U.S.A*, **12**, 4991; 1974). To finally prove that the larger molecule β is the actual reverse transcriptase it must be isolated and

characterised — a difficult task, as β is very labile. Proteolytic degradation products may still retain some enzymatic properties, especially response to synthetic templates — as is the case with the isolated avian breakdown product α.

If the β subunit is the real avian viral transcriptase, it might correspond with the single polypeptide murine viral enzyme. But this is so far unproven. Two properties of the murine enzyme bear a strong resemblance to those of the α subunit: its low affinity for templates and its rather low response to natural RNA templates, which can be dramatically stimulated by addition of primers. It may therefore very well be that the actual mammalian viral reverse transcriptase has not yet been identified.

Perhaps the question of what the viral reverse transcriptase looks like cannot be answered by isolating the enzyme from the virus. One may have to look instead at the intracellular state of the enzyme. This has been done by Gallo's group (Mondal, Gallagher and Gallo, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 1194; 1975), who found interesting size differences between the reverse transcriptase from virus-producing cells and that of extracellular virus particles : the intracellular one is larger. It is not yet clear whether the high molecular weight class is an aggregate of the low molecular weight form and there is no evidence to correlate the two size classes with the avian β and α molecules.

One remaining problem is the small size of the DNA product of the reverse transcriptase. Baltimore and Duesberg recently made some progress here by increasing the deoxyribonucleotide concentration in the *in vitro* reaction, but the large amounts of radioactively labelled precursors required will be too expensive for normal use. Perhaps addition of cellular ligases will improve the size of the DNA product. Another important achievement would be the synthesis of a DNA product which represents all regions of the RNA template equally well—not just a small region in large amounts.

Furthermore the role of the RNase H needs to be shown. Does it remove the RNA in order to allow double-stranded DNA synthesis to occur? Such a mechanism was suggested soon after its discovery and is now also incorporated in the Cooper and Wyke replication model (*Cold Spring Harb Symp. quant. Biol.*, **39**, 997; 1974) But other models, requiring a complicated interaction between cellular and viral enzymes, have been proposed and recently supported (Leis *et al.*, *J. Virol.*, **15**, 484; 1975) Cooper and Wyke point to another interesting question: How

is the RNA primer removed from the RNA template to allow transcription of the anti-primer region?

None of these unsolved problems has affected the great usefulness of the viral reverse transcriptase in identifying the presence or involvement of RNA tumour viruses—particularly in human neoplasias. One still hopes for a specific inhibitor in order to test its effect on tumour growth—inhibition of reverse transcription is the only known virus-specific step which would not affect normal cellular functions Here H. Temin might object: he believes a reverse transcriptase plays a part not only in viral replication but also in normal cell development, for example in gene amplification. So far there is only weak evidence in favour of this exciting idea—but Temin was right once before[1]

Androgens converted to oestrogens

from our Steroid Biochemistry Correspondent

ALMOST 20 years ago it was demonstrated that androgenic steroids could be converted to oestrogenic ones even in the absence of steroid-producing endocrine organs: administration of testosterone to adrenalectomised ovariectomised women increased the urinary excretion of oestrogens. Recent studies have been concerned with the sites where such an aromatisation may occur and with the possibility that even a low level of conversion has an important effect at those sites.

Most tissues, including liver, seem able to bring about this conversion. Breast fat can form oestrogens (Nimrod and Ryan, *J. clin. Endocr.*, **10**, 367–372; 1975) and both abdominal and axillary fat can convert androstenedione to oestrone. This conversion by adipose tissue is surprising as one might expect the lipophilic steroid to be protected from the active enzymes by dissolving in the fat. Even though the rate at which oestrogens are produced *in vitro* by adipose tissue is very low, the large proportion of adipose tissue in the body (up to 30%) may make this tissue an important source. Oestrogens may have an important role in the aetiology and treatment of breast cancer; a large proportion of tumours are known to be oestrogen dependent and contain oestrogen receptors. But fat from normal breast tissue and that from subjects with cancer seems to have identical aromatising ability. Although oestrone is the main product formed, small amounts of oestradiol are also obtained.

Oestrogens may have a role in hair

growth and the presence of the aromatising enzyme system in anagen hair roots has also been demonstrated recently *in vitro* (Schweikert, Milewich and Wilson, *J clin. Endocr ,* **40**, 413–417; 1975).

Diencephalic tissue from human foetuses (Naftolin, Ryan and Petro, *J clin Endocr.*, **33**, 368–370; 1971) or limbic areas of rat brain (Lieberburg and McEwen, *Brain Res.,* **85**, 165–170, 1975) are able to aromatise testosterone or androstenedione but such conversion is negligible when cerebral cortical tissue is used. Whereas the radioactivity associated with oestradiol accounted for less than 1% of the radioactivity in the whole homogenate of the limbic areas of rat brain, in the nuclei oestradiol radioactivity counted for almost half the total. Although oestradiol could have been formed peripherally and taken up by the brain, the evidence would seem to show that oestrogens can be produced from C-19 steroids such as testosterone and androstenedione.

Marked changes in reproductive function can be caused by the administration of either testosterone or oestrogens to neonatal rats. This and other evidence suggests that the conversion of androgens to oestrogens in certain areas of the brain may have important effects on brain differentiation, sexual behaviour and the production of pituitary hormones.

Dilatancy and aftershocks

from Peter J. Smith

DILATANCY models are generally thought of in connection with either earthquake precursors or man-made earthquakes such as those induced by fluid injection at Denver, Colorado, during the mid-1960s. And though not everyone would agree on precisely how and why the migrating pore fluids account for the phenomena attributed to them, it is clear that if dilatancy is applicable at all it must be applicable to seismic activity extending over at least tens of years (because the precursors of very large earthquakes appear several decades ahead of the main shock) But what part, if any, does fluid flow play in aftershock sequences which also occur within this time scale? The question was first investigated by Nur and Booker (*Science,* **175**, 885; 1972) some years ago. But in having another look at it, Robinson *et al* (*Geophys. J ,* **41**, 37; 1975) have made an unexpected and encouraging discovery—encouraging because it shows that dilatancy concepts may be adapted successfully to explain phenomena at least one step removed

from those for which they were originally conceived

The particular sequence studied by Robinson and his colleagues was that of the Inangahua, New Zealand, earthquake of 1968 (magnitude 7 1). The general properties of aftershock sequences, however, are already well known. Thus the frequency of aftershocks immediately after a large shallow earthquake is typically thousands a day and decays with time to perhaps ten or fewer shocks a day 100 days later Up to a year at least, the frequency is approximately inversely proportional to time and is related to magnitude (of the aftershocks themselves) through the well known Gutenberg–Richter equation in which log (frequency) is proportional to magnitude with a constant of proportionality b (the so-called b value) Geographically, aftershocks are closely related to the fault region of the main shock, and most of them have focal mechanisms similar to that of the main shock.

When Adams and Lowry (*New Zealand R. Soc. Bull.,* **9**, 129, 1971) investigated the Inangahua aftershocks a few years ago, they found them to be no exception to these general rules The b value was roughly unity, the shock frequency varied as $(time)^{-1\ 05}$, the epicentres lay within an elliptical area about $45\ km \times 25\ km$ extending SSW from near the main shock's epicentre, and both the main shock and most of the aftershocks were apparently due to thrust faulting But Adams and Lowry only looked at the first 40 days of the sequence Robinson *et al.*, by contrast, returned to study the sequence 3.6 years after it had begun, by which time the magnitude level had decreased to that of microearthquakes. What they found was that the b value was still about 1 (having remained constant for more than 3½ years and over a magnitude range of 5), that the shock frequency had continued to decrease with time in the same way (actually as $(time)^{-1\ 06}$) and that the new epicentres were distributed as the old (the elliptical area had contracted marginally) But the faulting had changed completely, from the dominant thrust movement of the main shock and early aftershocks to the main normal movement of the later aftershocks

On the face of it, this is surprising The regional tectonic stress is compressive and thus hardly conducive to normal faulting unless both the stress is reduced to a low level and material is removed at depth. The earthquake and its aftershocks themselves will reduce the stress, but what could remove the material? The obvious explanation, of course, is that the pore fluids which entered the source region before the earthquake have now flowed out again. In general terms, the

normal faulting associated with the later aftershocks will therefore be due to the subsidence following the outflow. In short, the change to normal faulting late in the aftershock sequence is apparently a natural consequence of the pre-earthquake dilatancy.

Nuclear friction

from P. E. Hodgson

MANY complicated interactions take place when two nuclei collide at high energies, so that it is impracticable to develop a detailed quantum-mechanical theory. Instead, it has been found possible to understand some of the more important qualitative features of these interactions by making use of classical concepts such as orbit theory, friction and the behaviour of drops of liquid.

Among the more notable features of the energetic collisions of heavy ions is the rather distinct division between interactions in which a few nucleons are transferred from one ion to the other, with little loss of energy, and the so-called 'strongly damped' collisions in which the ions lose a substantial fraction of their energy. For each type of interaction, particular scattering angles are favoured, depending on the energy of the interaction.

In some cases the strongly damped inelastic collisions have energy losses as high as 200 MeV even though the final nuclei are very similar to the initial ones. This suggests 'that the nuclei can become highly excited in the interaction without substantially losing their identities or their original trajectories. After the interaction it is found that the ions have significantly less energy than they would have gained from Coulomb repulsion alone if they had started from a position of rest with their surfaces just touching.

In addition to these direct reactions, it is always possible for the two ions to coalesce or fuse together to form a compound nucleus, and the cross section for this is substantially less than the total cross section.

Beck and Gross (*Phys. Lett.*, **47B**, 143; 1973) have proposed that these phenomena may be understood if we think of the nuclei as experiencing strong frictional forces as they move in each other's field. These frictional forces convert kinetic energy into internal excitation energy, and thus reduce their relative angular momenta. In some cases the loss of angular momentum is so great that the nuclei cannot separate again, and they fuse together. In other cases they succeed in separating but only after strong mutual excitation has taken place.

This theory has recently been extended by Bondorf, Huizenga, Sobel and Sperber (*Phys. Rev.*, **C11**, 1265;

1975), who give a more detailed account of the processes that occur when one ion collides with another at high energy. The mutual interaction of the two ions may be described by a potential with three terms; first, the repulsive electrostatic or Coulomb potential, equal to $Z_1 Z_2 e^2 / r$ outside the ions and rather less inside, where Z_1 and Z_2 are the charges on the ions and r their distance apart. Second, there is the strongly attractive nuclear potential that essentially acts only within the volume occupied by the ions themselves and falls off exponentially outside. Third, there is the centrifugal potential $l(l+1)/r^2$ that accounts for the increasing difficulty for ions with high relative angular momenta l to approach each other; it is therefore repulsive.

At high energies l is large and the centrifugal potential dominates, so the total potential depends on l and r in the way shown for two values of l in the figure. The potential is more strongly repulsive for higher l and falls off rapidly as r increases.

This potential describes the forces acting on one ion due to the other, and classically speaking determines their orbits during the interaction. As soon as the ions touch each other additional energy-dissipating forces come into play, and these are usually represented by an absorbing potential. Quantum-mechanically this can be included by allowing the potential to become complex, as an imaginary potential has the effect of absorbing an incident wave, just as the refraction and absorption of a light wave can be described by attributing to the medium a complex refractive index.

We are now in a position to describe what happens to one ion that encounters another ion with orbital angular momentum l. If l is high the potential prevents the ions from touching, and the ion is elastically scattered or possibly the ions excite each other through their Coulomb fields. As l is progressively reduced the ions come closer together and begin to interact through their nuclear fields as well. Nuclear inelastic scattering can take place, and also the transfer of a few nucleons from one ion to the other. All these processes involve rather little energy, as the ions separate again with nearly the same energies as they had before the interaction These processes are represented by the dashed line on the figure.

At slightly lower values of l the ions interact very strongly; this happens quite suddenly because the nuclear densities rise very rapidly in the region of the nuclear surface. There is thus a critical l_{SDC} (strongly damped collision) below which the ions interact strongly. They stick together and nearly all their
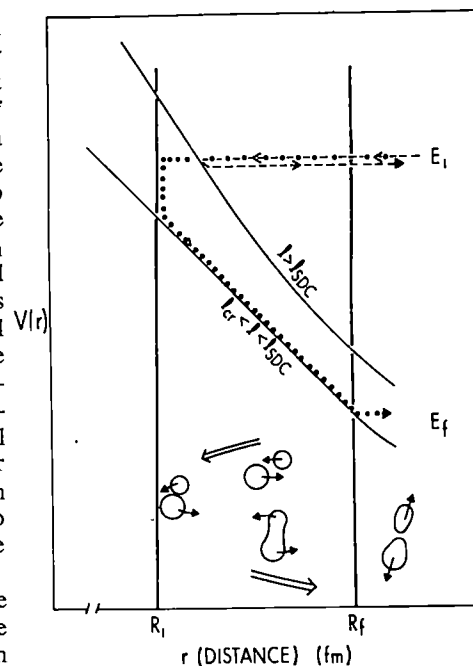
Schematic representation of the energy for strongly damped collisions. For $l > l_{SDC}$, the ions fail to reach the separation R_i at which they interact strongly, and therefore elastic scattering or few-nucleon transfer occurs. For $l_{cr} < l < l_{SDC}$ they reach R_i and the radial and rotational kinetic energies are suddenly dissipated. The ions stick together for a short while, then a neck develops due to the repulsive Coulomb force, retarded by radial friction. The sketches in the lower part of the figure indicate the shape of the system as the strongly damped collision develops.

radial kinetic energy and angular momentum is suddenly dissipated. If R_i is the radial distance at which the nuclei initially touch, then such ions follow the path shown by the dotted line in the figure. After a sudden loss of energy that brings the angular momentum down to a value l the joint system continues to rotate like a dumbbell and further energy is lost in mutual excitation. These processes are extremely complicated at the nucleon level but may be described in a very general way using the classical concept of friction.

At this point we make use of a familiar observation from the behaviour of colliding drops of liquid, namely that what happens when they approach each other is not the same as what happens when they separate. As they come together, they remain spherical and join together only when their separation is the sum of their radii. But when they separate a neck forms between them and gradually narrows as they separate, finally breaking only when the drops are separated by a distance substantially greater than the sum of their radii when spherical.

We assume that the same thing happens for colliding nuclei. As they begin to separate, a neck forms between them and energy is continually

lost. They finally separate only when their distance apart is R_f, which is substantially greater than R_1.

For even smaller l values, so much energy is dissipated by frictional forces that the nuclei stick together permanently and a compound nucleus is formed. The highest l value for which this occurs is the critical l_c for fusion of the two nuclei.

The cross sections for these processes can be expressed in terms of the angular momenta using the expression for the angular momentum

$$l\hbar = mvR = k\hbar R$$

So that the cross-section

$$\sigma = \pi R^2 = \frac{\pi}{k^2} l^2 = \pi \lambda^2 l^2$$

Thus the total cross section for strongly damped collisions

$$\sigma_{SDC} = \pi \lambda^2 (l_{SDC}^2 - l_{cr}^2)$$

and the cross section for compound nucleus formation

$$\sigma_{CN} = \pi \lambda^2 l_{cr}^2$$

This simple picture of the interaction of heavy ions at high energies explains in a qualitative way some of the features that have already been mentioned.

First, it is clear that for $l > l_{SDC}$, the energy loss is quite small, while for $l < l_{SDC}$, it is very large. This explains the prominent gap in the energy spectrum of the outgoing particles. Second, the formation of a neck means that the ions are separated by more that the sum of their radii when they part, and so will have less repulsive Coulomb energy, again in accord with measurements. Finally, the cross section for the formation of a compound nucleus is much less than the geometrical cross section.

As an indication of the value of l involved in a typical reaction, Bondorf and colleagues consider the collision of 600 MeV ^{84}Kr with ^{209}Bi and find l_{SDC} =250 and $l_{cr} \approx 75$; showing how large a number of partial waves participate in these reactions. They developed a formalism connecting these parameters of the interaction with the classical angles of emission of the particles at the critical angular momenta, and included a coefficient of friction k by assuming that the frictional force is proportional to the velocity of the ion. From the angles of the observed peaks in the cross-section they deduced values of k and found that it varies with l. In this way they were able to account in a qualitative way for the characteristic angles of emission of the particles losing little energy and of those losing most of their energy.

These ideas have also been worked out by Gross and Kalinowski (*Phys. Lett.* **48B**, 302; 1974) using the Newtonian equations of motion with frictional forces. They calculated the interaction potential experienced by the lighter of the two colliding nuclei by averaging the nucleon–nucleus optical potential over the density distribution of the heavier, and assumed a predominantly radial form for the friction tensor. With this simple model they were able to account for a large number of experimental fusion cross sections, and their energy variation.

This model will have to be tested for many reactions over a range of energies but already the results are sufficiently encouraging to show the value of applying the classical concept of friction to nuclear reactions. As in the corresponding macroscopic situation, we use the idea of friction to take account in a simple way of very complicated processes we do not attempt to describe in detail, and to build them into a more comprehensive picture of the whole interaction.

Linking lymphomas with immunodeficiency

from R. T. D. Oliver

"NOWHERE in pathology has a chaos of names so clouded clear concept as in the subject of lymphoid tumours" (Willis, *Pathology of Tumours*, 49, 760; 1948). Twenty-seven years and three classifications later we understand little more about the basis for the wide range of pathological entities classified as lymphomas. The many immunological deficiency diseases are similarly confusing. At first glance the article recently published in the *Lancet* on Duncan's disease (Purtilo *et al.*, *Lancet*, i, 935; 1975) (X-linked progressive combined variable immunodeficiency, to give its full name) seems to add yet another syndrome to the growing list. But more detailed reading suggests that it may provide clues for a better understanding of both immune deficiency syndromes and lymphomas.

Purtilo and colleagues report on studies of a family where six out of eighteen male children in seven branches of the family died of a lymphoproliferative disease involving bone marrow, liver, spleen and CNS. In three of these children it was possible to document an infection with infectious mononucleosis virus (Epstein–Barr virus) at presentation or prior to the onset of the disease and two had histologically proven lymphoma of the gastrointestinal tract and CNS. The authors suggest that infection with the Epstein–

Barr virus in the face of relative inability to respond to that virus triggered the fatal proliferation of lymphocytes (presumably B cells in view of the well-known ability of the Epstein–Barr virus to cause transformation *in vitro* of human B lymphocytes, though the authors fail to describe detailed immunological studies of the patients or their normal relatives). They suggest that the immune deficiency is mediated by a sex-linked recessive gene because exactly 50% of males but no females in the involved arms of the family tree died with the disease. This observation is extremely interesting as to date five out of fourteen previously recognised sub-groups of immune deficiency disease have been shown to have a sex-linked recessive inheritance (Fudenberg *et al.*, *Paediatrics*, **47**, 927; 1971) and it is well recognised that the majority of lymphoma except nodular sclerosing Hodgkin's and nodular lymphomas have an excess of males. But the generally low incidence of familial lymphomas clearly indicates that, if X-linked genes are involved, other genes must be also, as is well illustrated in the family studies of children with acute lymphoblastic leukaemia by Till *et al.* (*Br. J. Haemat.*, **29**, 575; 1975).

There is evidence, from studies in experimental animals, that at least four different classes of immune response gene exist. The first to be discovered was that linked to the *H*-2 major histocompatibility region, which seems to be involved in initial antigen recognition by T cells (Katz and Benacerraf, *Transplantn Rev.*, **22**, 175; 1975), and in resistance to various leukaemia viruses (Lilly and Pincus, *Adv. Cancer Res.*, **17**, 231; 1973). The second class, found in some cases linked to genes controlling immunoglobulin allotypes, regulates quantitative aspects of antibody production (Braun, Eichmann and Krause, *J. exp. Med.*, **129**, 809; 1969). The third class are the sex chromosome X-linked genes which, to date, have only been shown to influence response to T cell independent antigens (Mozes and Fuchs, *Nature*, **249**, 167; 1974). The fourth class recently reported by Jormalainen, Mozes and Sela (*J. exp. Med.*, **141**, 1057; 1975) seems to mediate its effect by some form of suppression of preordained response. If there were this number of different immune response genes in man which could influence the response of patients with lymphoma (even if the disease was induced by a single aetiological agent), it would hardly be surprising that no two lymphoma patients had exactly identical pathology in their lymph nodes.

It is clearly going to be important to be able to detect the different classes of immune response genes in man. A start has been made by classifying lymphomas on the basis of differentiation

T or B cell markers on their surfaces (Brown *et al.*, *Lancet*, **ii**, 753; 1974), and there have been many studies of HL-A antigens in patients with lymphomas; but we are far from understanding the complexity of their interaction with other systems such as the sex-linked locus postulated by Purtilo *et al. In vitro* assays, such as that recently reported by Greenberg, Gray and Yunis (*J. exp. Med.*, **141**, 935; 1975) will undoubtedly play an important part in this work.

Quark soup, with glue

from W. T. Toner

IF nuclear matter is made of quarks, so are neutron stars. If the quarks move freely as long as they remain close to each other in the neutron, then they must move freely in the centre of neutron stars where the density is a hundred to a thousand times greater than that of the neutron itself. So argue Collins and Perry in a recent issue of *Physical Review Letters* (**34**, 1353, 1975).

The fractionally charged quark is still the simplest realisation of the abstract logic shown by group theoretical analyses of the spectroscopic patterns made by more than a hundred strongly interacting particles. Although all models have their problems, a remarkably successful fit can be obtained by considering quarks which are light and move freely in a potential well. This might be thought coincidental were it not for the evidence of experiments in which leptons (electrons, muons and neutrinos) scatter from the nuclear matter of neutrons and protons. In violent collisions, these electromagnetic and weak probes measure the degree of concentration of electromagnetic or weak charge in the strongly interacting target: Δp is large and since $\Delta p.\Delta x \sim \hbar$, Δx is small. If the target is allowed to break up, its mass during and after the collision need not be precisely defined and ΔE in $\Delta E.\Delta t \sim \hbar$ can be large, Δt small. Violent inelastic collisions can thus be said to measure the instantaneous degree of concentration of charge. As is by now well known, the data show the large and 'scaling' cross section characteristic of the scattering from quasi-free point charges or 'partons'. The numerical values are most simply understood by assuming that the partons are the same fractionally charged, freely moving quarks that account for the spectroscopy. They seem free so long as we look quickly.

But our best efforts to produce quarks have failed, so how can they be thought to be real? There is no refuge in the abstractions of the algebra of currents since any theory which describes the data turns out to have the character of a free field

theory, and free fields mean free particles, or quasi-particles at the least.

The theorist is required to invent a glue to bind the quarks with the property that if they try to move too far apart the restoring force becomes very large, possibly infinite. But the closer they get together, the weaker it becomes: the quarks are asymptotically free. Such theories are being developed and some even have the very desirable properties of gauge invariance and renormalisability. Particle physicists are so used to forces which increase at short distances that they find such a behaviour peculiar and the talk of 'quark confinement bags' hard to swallow, although analogies in other branches of physics are not hard to find. Consider the electrons in a metal, liberated from the strong binding forces of their own atom by the proximity of others and able to migrate under the influence of infinitesimal potential differences until they reach the surface, where they meet a potential wall several volts high. Closer to home, there is the independent particle model of the nucleus.

If the long-range force binding quarks in elementary particles is not just large, but infinite, so that quarks can never be observed directly, it will be extremely difficult to verify an asymptotically free theory. The predictions are likely to depend as much on the values of the parameters as on the essential correctness of the ideas. For example, the simple prescription for electron–positron annihilation that the cross section is given by the sums of the squares of the quark charges is only valid in the asymptotic region, and where might that begin? It would be invaluable to have independent confirmation of the asymptotically free quark picture from a qualitatively different situation.

Collins and Perry argue that in the enormously dense matter at the centre of

---

A hundred years ago

IN an article in the July number of *Symons's Monthly Meteorological Magazine*, on the French floods, is an interesting calculation which will give Londoners some idea of what a "flood" means. Supposing we had a flood in the Thames, it would cover on the south bank, the whole of Battersea Park, Lambeth, Southwark, Bermondsey, and Deptford; and on the north bank, Fulham, Chelsea, Brompton, Belgravia, Westminster, and St. James's Park; while, as for the new embankment, a steamer might ply over the top of it.

from *Nature*, **12**, 261; July 29, 1875.

---

neutron stars the strongly interacting neutrons must overlap to such an extent that the strong, long-range forces—whatever their precise nature—will be screened and the quarks will no longer be confined to one group. They will interact rather weakly with each other and should move freely as if in a superdense gas or 'soup'. Since many of the properties of gases depend on correctly counting the number of independent states, their results differ from those of theories in which the neutrons and other composite strongly interacting particles make up the soup. Because the short-range forces which remain are weak, they should be calculable by perturbation (power series) methods. Detailed examinations of cosmological and astrophysical questions will be made in future papers.

Rabies

from Arie J. Zuckerman

THE two unassociated recent deaths from rabies in London underline the fact that even with modern intensive medical treatment there is little chance of recovery. Rabies is not declining in prevalence; on the contrary it is spreading, especially among wild animals (*Nature*, **242**, 228; 1973). The current outbreak in Europe is advancing in France along a front nearly 800 km long at a rate of about 30 km a year (*Nature*, **251**, 663; 1974).

Rabies virus is classified as a member of the rhabdovirus group, which includes a diverse collection of bullet-shaped RNA viruses from mammals, reptiles, fish, insects and plants. The virus particles contain two distinct major antigens, a glycoprotein from the virus membrane and an internal nucleoprotein antigen. The glycoprotein antigen elicits the formation of virus-neutralising antibodies which afford protection in animals against subsequent challenge with rabies virus. The same antibodies are probably also protective in man (*WHO Techn. Rep. Ser., No. 523;* 1973). Complement-fixing and precipitating antibodies to the nucleoprotein are devoid of virus-neutralising activity. The role of cell-mediated immunity in protection is as yet undetermined, but it may be crucial in the exposed individual.

Rabies virus is transmitted by contact, commonly through a bite. Human infection is usually acquired by bite but infection by aerosol has resulted from natural and laboratory exposure (the latter during vaccine preparation), and there is also some evidence of oral infection and aerosol contact in animals. The incubation period is 3 to 8 weeks. The virus spreads from the site of introduction to the central nervous system along the peripheral nerves.

Following infection of the central nervous system the virus may multiply in the salivary glands, in the kidneys and elsewhere and in some species in the muscle and the lungs.

Rabies is enzootic in all countries, except Australia and Antarctica, existing in two forms: urban rabies, propagated principally in dogs, and rabies in wildlife. Wild carnivores appear to be the main transmitters of epidemic and endemic wildlife rabies and methods of control have been reviewed in detail elsewhere (WHO Report, *loc. cit.*). Some progress has recently been made in the treatment and prevention of rabies in man. Ideally, before instituting treatment it is important to determine whether the biting animal was vaccinated and whether the animal was rabid. Local treatment of the wound is extremely valuable, especially when applied very early after exposure. This is followed by the combined administration of anti-rabies serum, preferably of human origin, and active immunisation. In many countries an immunisation schedule of 14 to 21 daily injections is being used and booster doses are essential when combined antiserum–vaccine treatment is employed A rabid patient should be isolated and treated in an intensive medical care unit.

The vaccines which have been used are of two types: those derived from the brain of adult or newborn animals and those derived from avian non-nervous tissue. Vaccine prepared from a Pasteur or similar strain of fixed virus grown in duck embyros has been developed in an attempt to eliminate the hazards associated with the brain tissue vaccine, which include neuroparalysis. Some progress has also been made in the purification of nervous tissue vaccines by extraction with fluorocarbon and other treatment. But even more promising are the vaccines produced in cell cultures. In the USSR and Czechoslovakia the Vrukovo-32 strain has been grown in primary hamster kidney cells, in France vaccine has been prepared in primary foetal bovine kidney cultures and in the USA and France an inactivated vaccine is being produced in human diploid cells with a Pasteur-derived PM strain adapted to cell culture. Three injections of this vaccine, given at intervals of 3 to 4 days in previously non-immunised subjects, elicit a neutralising antibody response which is much greater than that induced by 12 to 14 daily injections of either brain tissue or duck embryo vaccine (WHO Report, *loc cit.*). Indeed evidence of studies to date indicates that cell culture vaccines offer distinct advantages over existing vaccines of nervous tissue or avian origin, from the points of view of both safety and potency. Clearly careful assessment and evaluation of the newly developed cell

culture vaccines for man must continue. Pre-exposure immunisation of persons who run a high risk of repeated exposure, such as veterinarians and field naturalists, is highly desirable. Such immunisation consists of three injections of a potent vaccine at 5- to 7-day intervals followed by a booster injection a month later and subsequently at intervals of 1 to 3 years. Prophylactic vaccination of dogs and cats and other species where appropriate is most important, as are local administrative programmes and international control of transfer of animals. But the control of rabies in wildlife continues to challenge man's ingenuity.

Nuclear reactor in the jungle

from S. A. Durrani

An international symposium on the Oklo phenomenon was held at Libreville in Gabon on June 23–27, 1975, under the joint auspices of the International Atomic Energy Agency (IAEA) and the French Atomic Energy Commission (CEA), and sponsored by the Government of Gabon.

NATURE, it would seem, had anticipated man by something like 1,800 million years in bringing about the first self-sustained nuclear chain reaction on the Earth. And, contrary to common belief, it was not in the squash court of the University of Chicago in December 1942, but in the wilds of what is today the Republic of Gabon at a place called Oklo that this fantastic phenomenon took place.

The history of the discovery of the phenomenon, as it unfolded during the symposium, is briefly as follows. In June 1972, a team working under the direction of Dr H. V. Bouzigues at the CEA service laboratory at Pierrelatte in France noticed a marked anomaly in the abundance of the uranium-235 isotope (0 7171±0.0010 in atomic per cent instead of the normal 0.7202± 0.0006) during the certification of a secondary standard of UF_6 by the gas diffusion method. Later, much larger depletions of this isotope were discovered (down to 0.621%, and eventually to 0.296% [235]U) in uranium samples from this source, which was traced back to the Oklo deposit. First positive proof of the hypothesis that a natural chain reaction was responsible for the depletion of the fissile component was furnished by Mme M. Neuilly and co-workers of CEA through the measurement of the ratios of fission-product rare earths detected in the ore by the spark source mass spectrometry technique. Two simul-

taneous submissions by the above two groups on September 25, 1972, to the Proceedings of the Academy of Sciences, Paris, announced the discovery and the proposed explanation of this remarkable phenomenon It was pointed out that at the time of the reaction the natural abundance of the relatively fast-decaying [235]U isotope was more than 3%. This natural 'enrichment', helped by the moderation of the fission neutrons by the water content of the soil which enhanced their fission efficiency, and possibly by the relative absence of neutron-absorbing elements in the surroundings, allowed a nuclear chain reaction to develop. It is perhaps worth mentioning that such a natural chain reaction had already been predicted, on theoretical grounds, by several scientists, notably by P. K. Kuroda as early as 1956. The scientific secretary of the symposium, Dr R. Naudet of CEN, Saclay, has since late in 1972 been leading the 'Franceville Project' established by the French CEA to investigate the phenomenon, and has done a great deal to promote its study internationally.

One of the questions discussed at the conference (for example by E. Roth of CEN, Saclay) was whether the Oklo phenomenon was unique. The general feeling was that such propitious circumstances must have occurred elsewhere on the Earth. The timing (~1,800 Myr ago) may well have been optimal. The role of organic matter in producing 'super-concentrations' of uranium in the Franceville basin was delineated by J. Connan (SNPA-Pau, France). At earlier times, organic matter may not have been present in sufficient quantities, whereas more recently the [235]U component would have diminished. One intriguing possibility being discussed in the corridors of the conference was whether the radiation accompanying Oklo-type reactions over the globe could have played a role in the furthering of the evolutionary process. (It is believed by some palaeontologists that the origin of mitotic, nucleated cells lies somewhere between 1,500 and 2,000 Myr ago.)

The fact that most of the fission products seem to have stayed put over ~1,800 Myr has great relevance to present-day waste disposal problems in the field of nuclear reactors (and nuclear explosions). These implications were reviewed by R. D. Walton (Division of Waste Management and Transportation of USERDA) and by C. Fréjaques (CEA). The latter pointed out that [239]Pu seems to have hardly moved at all during its half-life of ~24,000 yr. Such conclusions, if confirmed, would be of profound importance in the future development of nuclear energy, both peaceful and destructive.

review articles

Archaeological evidence of environmental change

G. W. Dimbleby*

Pollen analysis, particularly combined with data from other animal, vegetable and mineral remains on archaeological sites, can yield much information on climatic conditions and the development of agriculture in prehistory. There remain, however, considerable problems of interpretation where the indications from different sources are at variance.

IT is now commonplace to read accounts of changing environmental conditions over periods of thousands of years, sometimes even going back into the earlier stages of the Pleistocene. This is largely due to the great development of pollen analysis, combined in later periods with radiocarbon dating, which has now been applied in many different parts of the world and to many different types of deposit. Evidence of climatic change is also indicated by other features such as change in sea levels or invertebrate faunas. In the more remote past these changes could only have been due to changes in climate and other secular factors determining the environment.

In later times, however, and especially in the past 10,000 years, man was operating within the landscape, using powerful ecological tools such as fire or agricultural practices. Some effect of these activities is often detectable in the pollen record of lake deposits or peat bogs; for instance, a reduction of tree pollen relative to herb pollen[1], or the appearance of weeds of arable land[2]. There are circumstances in which it is not easy to distinguish secular changes in the environment from the anthropogenic effects. For example, in the past 2,000–3,000 years of the postglacial period in Britain there has been a proportionate increase in the representation of birch (*Betula*) in the tree pollen totals[3]. This could be due to a progressive cooling of the climate, to a progressive acidification of soils, or it could be due to the influence of man in creating a more open landscape, and in particular with his use of fire to do so. What can rarely be told from pollen analyses is where changes were taking place and with what intensity.

It is here that ecological investigation of archaeological sites themselves can often fill in the picture. Once man started constructing earthworks, that is, from the Neolithic onwards, in doing so he was burying a sample of the contemporary land surface and so preserving it for posterity[4]. Even before then, in the Mesolithic, he sometimes created conditions which produced the same effect: wind blown sand resulting from forest destruction on occasion buried a contemporary land surface[5]. These buried surfaces, and to a lesser degree the material of the earthworks themselves, can be studied by some of the conventional methods, appropriately adapted, and also by other methods which are not applicable to the aquatic situations which are the usual source of evidence of past conditions.

Before I go on to consider these applied techniques in more detail, one important point must be made. In a peat bog,

preservation of pollen and other organic remains can be very good; moreover, such remains are well stratified, though perhaps not always as perfectly as is sometimes assumed. A buried land surface, however, can preserve material in no better condition that it was at the time of burial; and if stratification in the contemporary land surface was disrupted, as for instance by the activity of soil fauna, it will be preserved in the same disrupted state.

Pollen analysis

The value of pollen analyses of buried soils must depend on whether pollen analysis can be usefully applied to terrestrial soils. There is now quite an extensive literature on this subject[6-10], and it seems quite clear that acid soils can not only provide a surface humus layer which contains a contemporary pollen spectrum, but that the mineral soil beneath contains a record in broad terms of the vegetation history of the site, brought about by the progressive downwash of the older pollen into the deeper layers of the soil. This record may cover hundreds or even thousands of years.

Base-rich soils may contain no pollen, or at best small quantities. Such soils are often actively worked by earthworms and other soil animals, so that stratification is destroyed. It can be argued, however, that microbiological breakdown of pollen is rapid, so that all the pollen in the solum is more or less coeval. If so, the pollen of all the samples counted can be totalled to give a single pollen spectrum[11].

Using pollen analysis of buried soils it has been possible not only to show the progressive clearance of areas such as the North York Moors, but within any one area to show what types of land use were in operation in the various periods[12]. Even on calcareous soils clear evidence of open country in the early Neolithic has been obtained by this method[11], and it seems that it remained open from then onwards.

The pollen analyses of buried soils can sometimes be tied in with profiles from adjacent peat or lake deposits, but in other cases there is no apparent correlation. For example Thorley's pollen profile from Lewes[13] indicates that forest cover of the chalk extended until the Middle Bronze Age, yet the analyses of buried soils of Neolithic age may show open conditions[11]. Such discrepancies may arise from the fact that a pollen spectrum from a peat deposit is of a regional character whereas one from a buried soil is essentially local and not necessarily representative of the wider area.

Finally under this heading one should mention that pollen analyses of archaeological sites may contain artificial concen-

*Institute of Archaeology, University of London, 31-34 Gordon Square, London WC1, UK.

trations of pollen, such as the remarkable high percentages of ivy (*Hedera*) pollen found in some Mesolithic and later sites[14]. Such concentrations are unparalleled in aquatic deposits.

Other botanical material

Archaeological sites may contain other plant remains, some of which are valuable indicators of environmental conditions. Frequently these are charred, though in special circumstances, such as waterlogged deposits (wells and so on) or the peculiarly anaerobic conditions of Silbury Hill, good preservation of uncharred material may occur. For instance, under Silbury Hill[15], and in the waterlogged Wilsford Shaft, moss plants were found which were still green. They were readily identified and indicated the existence of chalk grassland turf in Neolithic and Bronze Age times.

In the early 1950s Helbaek[16] carried out his extensive investigation of seed impressions on prehistoric pottery, but since then the development of the flotation technique[17] has increased enormously the direct evidence not only of the crop plants but also of the associated weeds (see, for example, Dennell[18]). In the New World much evidence about early food plants and their associated weeds has come from cave deposits, including human and animal coprolites[19]. Such evidence has been of outstanding importance in the quest for the origins of maize[20].

Charcoal and sometimes uncharred wood may be found on many archaeological sites. They are of limited value as environmental indicators because wood was a material collected by man for fuel and for constructional purposes. Such finds are therefore usually only of general significance. Nevertheless interesting facts may arise; for example, in spite of the poor showing of *Pinus* pollen in Zone VIIb deposits of Southern England, the charcoal of *P. sylvestris* is recorded in a number of sites through the Neolithic and Bronze Age[21,22].

Other plant remains may be found using the appropriate techniques. Bud scales have been isolated and identified from waterlogged deposits[23] and grass phytoliths may occasionally be found in large concentrations[24]. The ecological value of such records remains to be seen.

Animal remains

Vertebrate bones are often an abundant feature of prehistoric sites with a high *p*H. The larger bones are valuable evidence of man's use of animals and of their structural features, but they are of limited value as ecological indicators—except in so far as domestic animals represent a powerful ecological influence.

The smaller vertebrates—rodents, amphibia, birds—have received relatively little study. These groups present some difficulties of identification, but their potential as environmental indicators is considerable. One source of their remains, found occasionally on archaeological sites[25], is the regurgitated pellets of birds of prey.

Invertebrate remains offer the greatest promise as environmental indicators, but they can also offer formidable problems of identification. Important results have been achieved with molluscs[26]. In calcareous soils they can provide evidence of changes of environment; for example, the sequence from forest through clearance to grassland has been demonstrated a number of times by Evans[4]. As with soil pollen, their distribution in the soil profile can also give a broad picture of preceding environmental change, though it is not entirely clear how a degree of stratification comes about in a calcareous soil. More work still needs to be done on the ecological requirements of the critical taxa, but it is obvious that they provide a tool of great value.

To an even greater extent is it true that more ecological knowledge is required about the insects. The Coleoptera in particular may be found in considerable numbers and variety of species[27,28] and although groups such as dung beetles may be over-represented, many other species must derive from the

locality and therefore be of particular ecological significance. Insect remains are also proving to be an important feature of urban sites where they can indicate the nature and use of the premises in which they occur[29].

It has been found that the exoskeletal remains of mites may be preserved in waterlogged archaeological deposits[30] and these, too, may be of considerable environmental significance. Our knowledge of the ecology of many species is still inadequate, but there have been considerable advances in this direction in recent years (see for example ref. 31).

Soils and sediments

This is a source of information which is rarely, and then only indirectly, available to those working on aquatic deposits. A buried land surface may provide evidence both of the ecological community and of the soil with which it was associated. By comparing the soil profiles buried beneath earthworks of different ages in the same area, it is possible to show the sequences of soil development, which culminates in the present-day soils[12]. It can be demonstrated that on acidic parent materials the clearance of primary forest, followed by pastoral or arable agriculture, may institute a new process of soil genesis, resulting in a different soil—a more acidic and less biologically active one—compared with the mature soil which preceded forest clearance. By combining pollen analysis with observations on the particle-size distribution in a profile it is sometimes possible to show at what stage in the process the earthworms of the original mull humus were eliminated by the soil acidification. In one site at Goodland in Northern Ireland[32], there is evidence that Neolithic clearance and farming led to soil acidification, loss of structure and eventually peat formation, a conclusion which accords with Moore's recent conclusions from the pollen analysis of blanket peats[33,34].

Even on calcareous soils there is some suggestion of soil change. Some of the Neolithic soil profiles are very shallow, as though truncated. Evans and Valentine[35] have evidence of hill-wash associated with initial clearance of scarp slopes. Certainly chalk soils eroded massively in late prehistoric times, as evidenced by the dry valley fill[36] and by lynchet formation[37].

Multi-disciplinary approach

Though few sites contain material in all the categories described above, most sites contain several different types of evidence. It is important that wherever possible more than one approach should be used. In some instances the different lines of enquiry corroborate each other completely. At Silbury Hill, for instance, the analyses of pollen, molluscs, insects, seeds, mosses and the soil profile all fitted in with a picture of pastoral agriculture in an open landscape. There are circumstances, however, where the different approaches seem to give contradictory results; one may suggest wooded conditions and the other grassland. In some cases these are readily explicable, as at Windmill Hill, where the Neolithic buried soil seems to have been truncated, so that the pollen and mollusc analyses apply to different phases[26]. In other cases no such explanation is obvious[11], and we have to consider more carefully the nature of the evidence. Pollen grains and snail shells are very different in nature; they get into the soil in different ways and their persistence in the soil depends on different processes. Enough work has been done on both materials to show that consistent results are obtainable with each, but discrepancies show that there are factors we have not yet taken sufficiently into account in building an archaeological interpretation from the analytical data.

Just as conventional pollen analysis was widely used before questions were asked about the relationship of pollen spectra to vegetation in modern environments, so the interpretation of archaeological environmental data is now at the stage of having to pause and consider the validity of the obvious conclusion

indicated by the evidence. We cannot turn to modern equivalents of early farming environments in regions like Europe—they no longer exist. Perhaps this is another area in which experimental archaeology may help us to solve our present problem.

1 Turner, J., *Proc. R Soc.*, B161, 343–354 (1965).
2 Sims, R. E., in *Quaternary Plant Ecology* (edit. by Birks, K. J. B , and West, R. G.), 223–236 (Blackwell, Oxford, 1973)
3 Pennington, W., *The History of British Vegetation.* (English Universities Press, London, 1969)
4 Evans, J G., in *Economy and Settlement in Neolithic and Early Bronze Age Britain and Europe* (edit. by Simpson, D. D. A), 27–73 (Leicester University Press, Leicester, 1971).
5 Dimbleby, G. W., *Proc. R. Soc.*, B161, 355–362 (1965).
6 Dimbleby, G. W , *J. Soil Sci.*, 12, 1–11 (1961).
7 Guillet, B., *Pollen et Spores*, 12, 45–69 (1970); 13, 233–254, 421–446 (1971).
8 Munaut, A. V., Durin, L., and Evrard, J. C , *Bull. Soc. bot. Nord France*, 21, 105–133 (1968).
9 Pertsch, R , *Landschaftsentwicklung und Bodenbildung auf der Stader Geest*, (Bundesforschungsanstalt fur Landeskunde und Raumordnung, Bad Godesberg, 1970).
10 Welten, M., *Veröff Geobot. Inst. Rubel Zurich*, 33, 253–274 (1958).
11 Dimbleby, G. W., and Evans, J. G., *J. Archaeol Sci.*, I, 117–133 (1974).
12 Dimbleby, G. W., *The Development of British Heathlands and their Soils* (Clarendon Press, Oxford, 1962).
13 Thorley, A., *Inst. Br Geogr Confr* , Part 5, 47–50 (1971).
14 Simmons, I G., and Dimbleby, G. W., *J. Archaeol. Sci* , 1, 291–296 (1974).
15 Williams, D., thesis, Univ Bradford (1975)
16 Helbaek, H , *Proc prehist. Soc* , 18, 194–233 (1953).
17 Jarman, H. N , Legge, A. J., and Charles, J. A., in *Papers in Economic Prehistory* (edit. by Higgs, E.), 39–48 (Cambridge University Press, London, 1972)
18 Dennell, R. W , *J. Archaeol. Sci.*, I, 275–284 (1974).
19 Callen, E. O., in *Science in Archaeology* (edit by Brothwell, D., and Higgs, E.), 235–243 (Thames and Hudson, London, 1969).
20 Mangelsdorf, P. C , MacNeish, R. S., and Galinat, W. C , in *Prehistoric Agriculture* (edit. by Struever, S), 471–486 (American Museum of Natural History, New York, 1971).
21 Cunnington, M. E , *Woodhenge* (Simpson, Devizes, 1929).
22 Ashbee, P , *Proc. Dorset nat. Hist archaeol. Soc.*, 80, 146–159 (1958)
23 Keepax, C., thesis, Univ. London (1972)
24 Dimbleby, G. W., *Plants and Archaeology* (Baker, London, 1967)
25 Jewell, P. A., *Advmt. Sci Lond.*, No 59, 165–172 (1958).
26 Evans, J. G., *Land Snails in Archaeology* (Seminar, London, 1972).
27 Osborne, P J., *J Anim Ecol.*, 38, 555–566 (1969)
28 Osborne, P. J , *Britannia*, 2, 156–165 (1971)
29 Buckland, P. C , *J. Archaeol. Sci.*, I, 303–316 (1974)
30 Harrison, S , thesis, Univ. London (1974).
31 Wallwork, J. A., *Ecology of Soil Animals* (McGraw-Hill, London, 1970).
32 Case, H J., Dimbleby, G. W., Mitchell, G. F., Morrison, M. E. S., and Proudfoot, V B., *J R Soc Antiq Irl.*, 99, 39–53 (1969)
33 Moore, P. D , *Nature*, 241, 350–353 (1973).
34 Moore, P. D., *Nature*, 250, 439–441 (1974).
35 Evans, J. G., and Valentine, K. W. G , *J. Archaeol Sci.*, I, 343–351 (1974).
36 Evans, J. G., *Proc. geol. Ass.*, 77, 347–364 (1966).
37 Fowler, P. J., and Evans, J. G., *Antiquity*, 41, 289–301 (1967)

Origin of blanket mires

Peter D. Moore*

There are three possible causes for the formation of the peat deposits which blanket parts of the British Isles and Scandinavia. Climate and soil maturation each plays a part, but the contribution of human activity now seems to be larger than had hitherto been supposed.

THE upland, oceanic regions of the western British Isles and Scandinavia have extensive areas covered by deposits of peat, which have developed over a wide range of substrate and topography. The peat blanket is most extensive over plateaux and gently shelving depressions but shallower on or absent from steeper slopes[1]. In depressions, the stratigraphic profile may reveal the presence of former lakes, dating from late-Devensian times[2], or the remains of damp woodland and carr[3,4] below the blanket peat cover. The age and origin of the blanket peats has been the source of much controversy, but evidence now accumulating links their origin with prehistoric human activity. Until recently, the possible influences of this factor had been underestimated in favour of those of climatic change and developmental processes within soil profiles.

The age of blanket peats

Early work on blanket peat stratigraphy was carried out by Conway in the southern Pennines[4,5], where extensive deposits exist. Her conclusion, based on pollen analysis, was that the peats began forming during Atlantic times (about 7,500–5,000 yr b.p.), which seemed reasonable in view of the generally wet conditions which then prevailed. Many of the shelving depressions examined by Conway, however, contained peat-forming woodland communities before the spread of true blanket peat. More recent work by Tallis[7] on the peats of slopes and plateaux suggest a later date, at about the time of the elm decline pollen horizon, although some of his higher altitude diagrams (for example, Featherbed Moss[8]) have basal deposits predating the elm decline.

Pollen analysis of blanket peats from other areas,

summarised in Table 1, show that peat initiation often occurred at about the time of the elm decline, the exceptions being mainly the deeper peats of the southern Pennines. Smith and Pilcher[9] have recently collated radiocarbon dates of the elm decline and other widely used Flandrian pollen zone horizons to examine the basic assumptions of geographical synchroneity of vegetational change, which underlies the general application of the Godwin[10,11] zonation system. They found that the dates of most pollen zone boundaries differ considerably according to the geographic position of the site, whereas the elm decline horizon remains remarkably synchronous at about 5,300–5,100 yr b p. Whatever the cause of the elm decline, whether climatic change, prehistoric human activity[12] or disease[13], it seems to be a reliable guide to the date of origin of many blanket peats, at about or just before 5,000 yr b.p. Caution is required, however, in interpreting shallow blanket peat profiles, the base of which may coincide with a secondary elm decline[14]. Profiles from the Highlands of Scotland are difficult to interpret because of the indistinct nature of the elm decline in these areas[15].

Bartley[16] has published a radiocarbon date of $5,490 \pm 140$ yr b.p. from the base of a blanket peat profile on Rishworth Moor, West Yorkshire, which coincides with the elm decline This is slightly older than is normal for this horizon, but peat accumulation is likely to have been slow at the time and the resolution of ^{14}C dating therefore poor.

The elm decline is itself usually accompanied by palynological indications of human activity and forest clearance, but even in the shallow peat sites, which were initiated later than the primary elm decline, the basal deposits often give indications of considerable human activity[14,17]. It is this coincidence of peat initiation with evidence for human interference with vegetation which makes imperative a reappraisal of the generally assumed climatic origin of blanket mires.

*Department of Plant Sciences, King's College, 68 Half Moon Lane, London SE24 9JF, UK.

Table 1 Blanket peat profiles and the relative position of the elm decline horizon to the peat–soil interface

Area	Site	Authors	Peat depth (cm)	Distance of elm decline from peat base
Dartmoor	Taw Head	Simmons[18]	390	10 cm below
Dartmoor	Okement Hill	Godwin[18]	155	5 cm above
Exmoor	Chains	Merryfield and Moore[14]	290	5 cm above
Mid-Wales	Plynlimmon	Moore[19]	185	10 cm above
Mid-Wales	Tywi Valley 5	Moore[4]	120	3 cm above
Mid-Wales	Tywi Valley 4	Moore[4]	65	0 cm
South Pennines	Down Head Hill I	Tallis[7]	150	0 cm
South Pennines	Down Head Hill II	Tallis[7]	185	30 cm above
South Pennines	Featherbed Moss	Tallis and Switsur[8]	260	45 cm above
South Pennines	Totley Moss	Hicks[20]	240	40 cm above
South Pennines	Hipper Sick	Hicks[20]	170	15 cm above
South Pennines	Ringinglow C	Conway[5]	600	50 cm above
South Pennines	Ringinglow A	Conway[5]	400	20 cm above
South Pennines	Ringinglow H	Conway[5]	120	20 cm above
South Pennines	Rishworth Moor	Bartley[16]	190	10 cm above
Lancashire	Deep Clough	Tallis and McGuire[21]	90	2 cm below
North York Moors	Lady Bridge Slack	Simmons[22]	310	15 cm above
Lake District	Red Tarn Moss	Pennington[23]	150	7 cm above
North Pennines	Coom Rigg B	Chapman[3]	150	0 cm

Conditions and causes of peat formation

Peat accumulates when the total respiratory activity of producers, consumers and decomposers fails to attain the same level as primary productivity. In fact it is the depression of decomposition rates which is most critical and this is related to waterlogging. Any environmental change which leads to increased waterlogging will therefore favour peat initiation. The three main variables which could be invoked to account for blanket peat formation are climate, soil maturation and human clearance of vegetation.

Frenzel[24] has argued the case for a short period of cold climate in many parts of the Northern Hemisphere between 5,400 and 5,000 years ago. This is disputed by Smith[25] who considers that much of Frenzel's data could equally be attributed to human activity, especially in upland areas. Lamb et al.[26] suggest that both rainfall and excess drainage water in Britain actually decreased by about 10% between 4000 and 2000 BC. Sources of evidence for climatic changes during this time are few, and often themselves of a botanical nature, for example, peat growth rates[27], and such evidence is open to alternative interpretations Iversen's[28] use of ivy, mistletoe and holly as climatic indicators on the Continent and its subsequent criticism by Troels Smith[29] on the grounds of prehistoric man's interest in these plants is a well known example of the misinterpretation of botanical evidence which is possible during this complex and confused period. It is generally agreed that Flandrian tree lines reached their maximum altitude at this time[30,31], but their subsequent retreat cannot necessarily be regarded as an entirely climatic effect. Independent evidence for climatic change at the time of the onset of blanket peat formation is, therefore, not adequate to allow quantification.

Blanket peat could be regarded as the terminal stage in soil maturation for high rainfall areas, in which case its formation is essentially pedogenic. Godwin (in ref. 30) took his position, considering podsolisation in high precipitation/evaporation conditions a prerequisite for peat formation.

Dimbleby[32], however, considers this unlikely because sub-peat soil profiles are frequently immature and do not display fully developed podsolic characteristics. Since many blanket peats are developed over soils which, though presumably waterlogged, do not give the impression of having suffered intense leaching[33], it is unlikely that podsolisation, pan formation and resultant drainage impedance is the main causative factor in peat initiation. In some Irish sites[34,35], Mitchell has stressed the role of pan formation as a cause of waterlogging, consequent on prehistoric ploughing. Often the depth of the thin pan corresponds with the depth to which the plough penetrated. Human land use may, therefore, be intricately connected with soil processes before peat initiation at some sites, but this explanation cannot be accepted for the majority of upland peats in Britain, where neither ploughing nor podsolisation necessarily precede peat initiation. Nor is it likely that such soil processes, even in worsening climatic conditions, could be responsible for peat initiation in the absence of human forest clearance.

For many years circumstantial evidence has been accumulating which links prehistoric human activity with the initiation of blanket peat deposits[18,35]. Dimbleby[37] showed that forest could be converted into moorland as a result of Bronze Age forest clearance and Simmons[18] suggested that even Mesolithic communities could have initiated such changes in Dartmoor. Simmons[38,39] has attempted to construct an explanatory model which might account for these vegetational changes, concentrating particularly on the capacity and the motives of Mesolithic populations for producing them. He concludes that their ecological impact in upland Britain would have been small, but that their relationship with red deer as an important food resource would have provided them with ample motive for upland clearance. Neolithic peoples, complete with truly domesticated herbivores, must have had even stronger motives for upland forest clearance and the general use of such areas for grazing.

Table 2 Summary of data concerning increased stream discharge following the deforestation of a watershed

Site	Forest type	Increased discharge on felling (%)	Author
South Appalachians, USA	Oak/hickory	15	Swank and Douglas[41]
Colorado, USA	Aspen, mixed conifer	22	Bates and Henry[42]
Colorado, USA	Lodgepole pine	25 (after 40% clearance)	Hoover and Leaf[43]
Japan	Mixed deciduous/conifers	8–24	Nakano[44]
New Hampshire, USA	Maple/beech/birch	40	Bormann and Likens[45]

The vital question which must be asked, however, is what would be the hydrological consequences of forest modification, since peat initiation is most closely linked with soil hydrology. The question is particularly relevant to blanket peat formation since the topographical features of the sites involved are often water-shedding rather than receiving. Removal of a tree canopy from such a situation would increase the supply of ground water by reducing the transpiration demand of the vegetation and also by reducing the water interception of the canopy, much of which would evaporate once more without reaching the ground. The considerable body of experimental data now available permits the estimation of water gains resulting from forest clearance. Table 2 is a summary of data derived from experimental deforestation of watersheds bearing a variety of forest types.

There are many problems associated with the interpretation of such figures and extrapolation from them to other situations[46]. In particular one should be mistrustful of data from watersheds where there has been inadequate calibration before felling; one must also consider the forest types involved, since coniferous trees intercept more water than deciduous ones[40]; and one must take into account the climatic conditions in which the data were obtained, particularly those factors influencing evaporation. The figures obtained by Swank and Douglas are perhaps the most acceptable in terms of rigorous calibration, but in the context of a more oceanic Britain during Neolithic times, with lower evaporation rates, a figure of 10% increased discharge following clearance would be a more reasonable estimate. Studies of soil water under different vegetation types[47] and direct studies of interception losses in forest stands[48] in Hampshire, England, confirm that 10–20% more water would be available on removal of a forest canopy.

Clearcutting of forest in Finland has been reported as causing a rise in water table of 38 cm (ref. 49), and thinning of a beech forest in Denmark has resulted in the death of remaining trees by waterlogging as a consequence of the higher water table produced[50]. It is this kind of effect which leads to the use of fire as a means of watershed management in the USA[51,52]. There are even some situations in which the clearance of a catchment can be directly correlated with peat development in the areas receiving runoff. For example, Skarzynska[53] has described such a situation in Poland where settlements of the Luzyce culture (Iron Age) in low lying areas were literally swamped by peat-forming plant communities as a consequence of the deforestation of the surrounding catchment. In more recent times, alder/spruce woodlands in Czechoslovakia have been converted to waterlogged meadows following clearance[54].

Having established that human clearance of woodland (by felling or fire) or even woodland thinning (by selective felling or free grazing by sheep or goats[55]) could modify hydrological regions very substantially, one must consider which prehistoric cultures may have been responsible for peat initiation in different parts of Britain. In some areas the influence of Mesolithic man in the uplands was considerable and Simmons[18] has implicated these cultures in his explanation of habitat changes on Dartmoor. Pennington[56] has described evidence for increased runoff in parts of the Lake District in late Mesolithic times and also for the development of blanket peat over mor humus and mineral soils during Neolithic times. Many radiocarbon dates for the early Neolithic are older than 5,000 yr b.p. (ref. 57) and give weight to the argument that many of the deeper, high-level blanket peats of western and northern Britain could have been initiated as a result of the activities of these peoples. Such activity may have involved no more than the free grazing of their stock coupled with occasional firing. In Ireland, the

extensive work carried out by Goddard[58] and Smith[59] suggests that, although some high altitude blanket peats began their formation during Neolithic times, the majority of peats studied were initiated in Beaker and Bronze Age times. Shallower and lower altitude peats on Exmoor[14] began to form during the Iron Age and during later times depending on local conditions of topography, climate and human land use, with the last-mentioned often acting in a critical manner to effect the crossing of the hydrological threshold for peat formation.

Three main conclusions emerge from these data. In the first place, prehistoric man was theoretically capable of influencing catchment hydrology in ways which would create effects of magnitude similar to those derived from major climatic changes. Second, circumstantial evidence from the dates of blanket peat initiation and from pollen assemblages at the base of these deposits, indicates man's involvement. Regional, topographical and altitudinal metachroneity is to be expected. This reflects the differing intensities of man's activities experienced in various areas and also the intensity of activity required for peat initiation at different sites. Finally, the soil changes associated with peat initiation are most satisfactorily explained as a consequence rather than a cause of the demise of forest and the development of peat.

1 Taylor, J. A., and Tucker, R. B. *Proc third Int Peat. Congr Quebec.*, 163–173 (1968).
2 Moore, P. D., and Chater, E. H., *New Phytol*, 68, 183–195 (1969).
3 Chapman, S. B., *J. Ecol.*, 52, 299–313 (1964)
4 Moore, P. D., *Proc fourth Int. Peat Congr Helsinki*, 1, 89–100 (1972).
5 Conway, V. M, *J. Ecol.*, 34, 149–181 (1947)
6 Conway, V M., *J. Ecol.*, 42, 117–147 (1954)
7 Tallis, J H, *J. Ecol.*, 52, 333–344 (1964).
8 Tallis, J. H., and Switsur, V. R, *J. Ecol.*, 61, 743–751 (1973).
9 Smith, A. G, and Pilcher, J R, *New Phytol*, 72, 903–914 (1973).
10 Godwin, H, *New Phytol*, 39, 370–400 (1940).
11 Godwin, H., *The History of the British Flora* (Cambridge University Press, London, 1956).
12 Smith, A G., *Proc. Linn. Soc Lond.*, 172, 38–49 (1961).
13 Watts, W. A., *Proc. Linn. Soc. Lond.*, 172, 81 (1961)
14 Merryfield, D L, and Moore, P. D, *Nature*, 250, 439–441 (1974).
15 Durno, S. E., and McVean, D. N., *New Phytol.*, 58, 228–236 (1959)
16 Bartley, D. D., *New Phytol*, 74, 375–381 (1975).
17 Moore, P D., *Nature*, 241, 350–353 (1973).
18 Simmons, I. G., *New Phytol.*, 63, 165–180 (1964).
19 Moore, P. D., *Nature*, 217, 1006–1009 (1968).
20 Hicks, S. P, *New Phytol.*, 70, 647–667 (1971)
21 Tallis, J H, and McGuire, J, *J. Ecol*, 60, 721–737 (1972)
22 Simmons, I. G., *New Phytol*, 68, 807–827 (1969)
23 Pennington, W., *Proc. R. Soc*, B161, 310–323 (1965)
24 Frenzel, B., in *World Climate from 8,000–0 B.C.* (edit by Sawyer, J. S.), 99–123 (Royal Meteorological Society, London, 1966).
25 Smith, A. G., in *Studies in the Vegetational History of the British Isles* (edit. by Walker, D., and West, R. G.), 81–96 (Cambridge University, London, 1970).
26 Lamb, H. H, Lewis, R. W. P., and Woodroffe, A, in *World Climate from 8,000–0 B C* (edit. by Sawyer, J S), 174–217 (Royal Meteorological Society, London, 1966)
27 Lamb, H. H., *Phil Trans R. Soc.*, A276, 195–230 (1974).
28 Iversen, J., *Geol. Foren. Forhandl*, 65, H3 (1944).
29 Troels-Smith, J., *Danm. Geol Unders* IV.R 4, No. 4 (1960).
30 Lamb, H H., *Q. Jl R. Met. Soc.*, 90, 382–394 (1964).
31 Pears, N. V., *Trans. Bot. Soc Edinb.*, 40, 536–544 (1969).
32 Dimbleby, G W., *Proc. R. Soc*, B161, 355–362 (1965).
33 Crampton, C B., *Scot Geogr. Mag.*, 82, 46–52 (1966).
34 Case, H. J, Dimbleby, G. W., Mitchell, G. F., Morrison, M. E. S, and Proudfoot, V. B., *J. R. Soc Ant. Irel.*, 99, 39–53 (1969).
35 Mitchell, G. F, *Proc. 24th. Int. Geol. Congr. Montreal*, Symp. 1, 59–68 (1972).
36 Watson, J. W., *Scot Geogr. Mag.*, 55, 148–161 (1939).
37 Dimbleby, G. W., *New Phytol.*, 51, 349–354 (1952)
38 Simmons, I. G., in *The Domestication and Exploitation of Plants and Animals* (edit. by Ucko, P. J., and Dimbleby, G. W), 111–119 (Duckworth, London, 1969).
39 Simmons, I. G., *J. Archaeol. Sci.*, 2, 1–15 (1975)
40 Dimbleby, G. W, and Simmons, I G., *J. Archaeol. Sci.*, 1, 291–296 (1974)
41 Swank, W T., and Douglas, J. E., *Science*, 185, 857–859 (1974).
42 Bates, C. G, and Henry, A. J., *USA Mon. Wea. Rev. Suppl*. 30, 1–79 (1928).
43 Hoover, M. D., and Leaf, C. F., in *Forest Hydrology* (edit. by Sopper, W. E, and Lull, H W.), 213–224 (Pergamon, Oxford, 1967).
44 Nakano, H, in *Forest Hydrology* (edit. by Sopper, W. E., and Lull, H W), 551–564 (Pergamon, Oxford, 1967).
45 Bormann, F. H., Likens, G. E., Fisher, D. W., and Pierce, R. S., *Science*, 159, 882–884 (1968).
46 Hibbert, A. R., in *Forest Hydrology* (edit. by Sopper, W. E., and Lull, H.W.), 527–550 (Pergamon, Oxford, 1967).
47 Rutter, A. J., and Fourt, J. appl. *Ecol.*, 2, 197–209 (1965).
48 Rutter, A. J, Morton, A. J., and Robens, P. C., *J appl. Ecol* 12, 367–380 (1975).
49 Heikurainen, L., in *Forest Hydrology* (edit. by Sopper, W. E., and Lull, H W.), 345–354 (Pergamon, Oxford, 1967)
50 Holstener-Jorgensen, H., in *Forest Hydrology* (edit by Sopper, W. E., and Lull,.H W.), 325–333 (Pergamon, Oxford, 1967)
51 Burma, G. D, *Proc. seventh Ann. Tall Timbers Fire Ecol. Conf.* 235–243 (1967).
52 Zwolinski, M. J., and Ehrenreich, J. H, *Proc. seventh Tall Timbers Fire Ecol. Conf.*, 195–205 (1967).
53 Skarzynska, K., *Bull. de l'Acad. Polon des Sci. Ser Geol et. Geogr.*, 13, 241–7 (1965).
54 Rybnicek, K., and Rybnickova, E., *Folia Geobot Phytotax.*,*Praha*, 9, 45–70 (1974).
55 Adams, S N., *J. appl. Ecol.*, 12, 143–152 (1975).
56 Pennington, W., *C.B.A. Res. Rept. No. 11*, 74–86 (1975).
57 Smith, I. F., in *British Prehistory: A New Outline* (edit by Renfrew, C.), 100–136 (Duckworth, London, 1974).
58 Goddard, A., thesis, Queens Univ. Belfast (1971).
59 Smith, A. G., *C.B.A. Res. Rept. No. 11*, 65–74 (1975).

articles

Chandler Wobble, earthquakes, rotation, and geomagnetic changes

Frank Press & Peter Briggs

Department of Earth and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

A pattern recognition algorithm can be applied to the seismicity of major earthquake belts, to the amplitudes of Chandler Wobble, to changes in the rotational velocity of the Earth, and to the drift of the eccentric geomagnetic dipole for the years 1901–64. The patterns which emerged suggest that all of these diverse phenomena are related.

THE causes of the Chandler Wobble and of variations in the length of the day remain a subject of speculation. The suggestion that movements accompanying great earthquakes excite the wobble faces the difficulty that the displacements may not be large enough[1,2]. But large, slow deformations (over hours or days) can occur in the epicentral area before and after a major failure and when these movements are added to the seismic slip that occurs with the main shock, the combined displacements seem to be large enough to excite the Chandler Wobble[3]. Short term changes in the length of the day (over periods of about 2 yr) have been related to variations in zonal wind velocity[4] and long term changes (over periods of about 10 yr) have been attributed to motions in the fluid core[5,6]. Presumably, the motion of the eccentric dipole is related to fluid motion in the core, and the compensatory changes required to conserve angular momentum of the Earth show up as changes in the length of the day.

Anderson has suggested that volcanism and climatic changes may be related to all of these phenomena[7]. According to his hypothesis, major volcanic eruptions lead to zonal wind changes which affect the rotation of the Earth, both on a short and long term basis. Small perturbations in the velocity of rotation trigger the release of the large amount of elastic energy stored in the lithosphere because of rotational processes, setting off global seismic activity and exciting the Chandler Wobble. The main supporting argument is the apparent correlation between global seismicity, fluctuations in rotation and amplitudes of the Chandler Wobble, and the indication that short term zonal wind changes seem to affect the rotation rate.

Here we apply a method of pattern recognition (ref. 8 and I. M. Gelfand *et al.*, unpublished) to see if we can detect a pattern in these diverse phenomena.

Pattern recognition algorithm

In a learning phase the computer is told into which of two groups—G_1 (26 yr) and G_2 (38 yr), associated with the amplitude of the Chandler Wobble and its decay, respectively—the build-up of data are to be placed for each of the 64 years from 1901 to 1964. Chandler Wobble data smoothed by taking 6-yr running averages are used[9].

For each year a series of heuristic questions is posed (Table 1) and answered yes/no, or 1/0 in binary code. The specific parameters for each question are assigned following a preliminary survey to obtain the best separation between G_1 and G_2. For example, for Question 16 (Table 1), many time windows were tested but the values used (present year) gave the best discrimination in that 73% of G_1 and only 10% of G_2 received a yes answer.

Characteristic traits for G_1 and G_2 years

The answer matrix consisting of the binary answers to the questions for each year (as well as a third category for unanswerable questions) is then examined to see if certain patterns emerge which characterise G_1 or G_2 years. A trait is a combination of answers to questions taken three at a time, two at a time or one at a time (triplet, doublet or singlet traits, respectively). For the 22 questions in Table 1 there are 13,288 possible traits. If a trait appears more than K_1 times in G_1 and less than $\tilde{K}_1$ times in G_2 it is identified as a characteristic trait for G_1 years. Similarly constants K_2 and $\tilde{K}_2$ are used to identify characteristic traits for G_2 years. For the questions cited in Table 1 the values $K_1 = 14$; $\tilde{K}_1 = 2$; $K_2 = 27$; and $\tilde{K}_2 = 2$ were used to obtain a few, in this case eight, independent characteristic traits for both G_1 and G_2. In a sense, we have used pattern recognition as an hypothesis selector and have checked thousands of possible hypotheses against data.

In a final step or predictive phase, a vote is taken for each year, with each G_1 characteristic trait assigned a positive vote and each G_2 characteristic trait a negative vote. For each year, the algebraic sum of G_1 and G_2 characteristic traits (the 'netvote') is used in most applications to predict future events[8], for example, to predict future build-ups or decays in the amplitude of the Chandler Wobble. These procedures may seem arbitrary, but pattern recognition lacks a formalism, as yet, and specific methods are justified by control experiments and other tests (I. M. Gelfand *et al.*, unpublished). The agreement between the netvote of characteristic traits and the Chandler Wobble amplitude data for the years 1901–64 is excellent and shows that once the computer has learned which are the characteristic traits, it can recognise and separate the years of build-up and decay of Chandler Wobble quite well using those traits alone.

It may, however, also be possible to discriminate between G_1 and G_2 years even if the questions were based on spurious data or random answers. In a control experiment we ran the entire program, including the preliminary one-dimensional survey, with spurious data obtained simply by reversing the time sequence of the actual data, that is, the substitution of 1964 for 1901 and so on, in obtaining answers to the questionnaire (Table 1). For the same K constants used with real data,

Table 1 Questions posed for pattern recognition

1 In the preceding 5th, 4th, or 3rd year WTAEO* $\geqslant 1.0$ EM-8† in the New Guinea–New Hebrides region? (Seismicity data from ref.10 and L. Knopoff, unpublished.)
2 In the following 0th, 1st, 2nd year WTAEO $\geqslant 1.0$ EM-8 in the New Guinea–New Hebrides region?
3 In the following 0th, 1st, 2nd, 3rd, or 4th year WTAEO $\geqslant 0.5$ EM-8 in the Tonga–Kermadec region?
4 In the preceding 2nd, 1st, or 0th year WTAEO > 1.5 EM-8 in the Kurile–Japan–Mariana region?
5 In the preceding 1st, 0th or following 1st year WTAEO > 2.0 EM-8 in the Peru–Chile region?
6 In the preceding 3rd, 2nd, 1st, 0th or following 1st year WTAEO > 1.0 EM-8 in central America?
7 In the preceding 2nd, 1st or 0th year WTAEO > 0.8 EM-8 in Java?
8 In the following 0th, 1st, 2nd, 3rd, or 4th year WTAEO > 1.5 EM-8 in the Ryukyu–Philippines region?
9 In the following 0th, 1st, 2nd, 3rd, or 4th year WTAEO > 0.5 EM-8 in the San Andreas–Gorda Ridge region?
10 In the preceding 2nd, 1st, 0th, or following 1st or 2nd year WTAEO > 0.5 EM-8 in China?
11 In the preceding 2nd, 1st, or 0th yar WTAEO > 0.5 EM-8 in the Aleutians?
12 In the preceding 1st, 0th, or following 1st year WTAEO > 0.5 EM-8 along a continent–continent collision zone?
13 In the preceding 5th, 4th, 3rd, 2nd, 1st, 0th, or following 1st year WTAEO > 0.5 EM-8 in the Caribbean?
14 In the preceding 1st, 0th, or following 1st year were there > 15.0 EM-8 integrated over the whole Earth?
15 In the preceding 7th, 6th, 5th, 4th, 3rd, 2nd, or 1st year was there a change in the absolute value of Earth's rotational acceleration ($|\dot{\omega}/\omega|$) of $> 2 \times 10^{-9}$ in units $\Delta\omega/\omega$ yr^{-1} (ref. 11)?
16 In the present year was the absolute value of |the magnitude of Earth's rotational acceleration ($|\dot{\omega}/\omega|$) $< 1.0 \times 10^{-9}$ in units $\Delta\omega/\omega$ yr^{-1}?
17 In the present year was the Earth's rotational acceleration negative?
18 In the present year was the length of day (l.o.d.) more than 0.5 ms longer than the mean value (corrected for secular slowdown)[11]?
19 In the following 2nd year was the speed (v) of the westward drift of the eccentric geomagnetic dipole > 0.25 degrees yr^{-1} (ref. 5)?
20 In the following 3rd or 4th year was the acceleration ($\dot{v}$) of the eccentric geomagnetic dipole motion negative?
21 In the preceding 1st year was the second derivative of the squared amplitude of the Chandler Wobble positive?
22 In the preceding 5th, 4th, or 3rd year was there a major volcanic eruption on Earth (as defined in ref. 12)?

*WTAEO means 'was there an event of'.
†EM-8 signifies a measure of seismicity in units of equivalent magnitude 8 earthquakes, obtained by dividing the cumulative, annual seismic moment released in a region by the seismic moment for a magnitude 8 earthquake.

G_2 characteristic traits could not be found. Only by relaxing the K constants could we obtain a separation of G_1 and G_2 years as measured by the netvote, and even then more years were misidentified than was the case for real data.

Moreover, the traits based on the spurious data did not assemble into coherent hypotheses relating seismicity, Earth rotation and geomagnetic field drift as did the real data. It is, therefore, reasonable to expect that the characteristic traits found with real data may carry physical information, although we cannot prove this.

To test the relationship between earthquake activity and Chandler Wobble excitation a pattern recognition run was made using the first 14 questions only. Questions 1–13 refer to earthquakes in individual seismic belts and Question 14 is a measure of world seismicity. Table 2 shows the traits characteristic of G_1 years of increasing Chandler Wobble amplitudes. Table 3 shows the characteristic traits for G_2 years of declining Chandler Wobble amplitudes. The striking pattern which emerges is that 11 of the 13 seismic belts show either major earthquake activity associated with years of increasing amplitudes or no activity for years of decreasing amplitudes. The

Table 2 Characteristic traits for G_1 years

Trait	Question													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14*
1	1	1	.	0	.	.	.	.	.	.	.	.	.	.
2	1	1	.	.	.	1	.	.	.	.	.	.	.	.
3	1	.	.	.	.	.	.	.	.	.	1	.	.	.
4	.	1	.	.	.	.	.	.	.	.	1	.	.	.
5	.	.	1	0	.	.	.	.	.	0	.	.	.	.
6	.	.	1	.	.	.	.	1	.	.	.	.	.	0
7	.	.	.	0	1	.	.	.	.	.	.	.	.	.
8	.	.	.	0	.	.	1	.	.	.	.	.	.	.
9	.	.	.	.	1	1	.	.	.	.	.	.	.	.
10	.	.	.	1	.	1	.	.	.	.	.	.	.	.
11	.	.	.	1	.	.	.	.	.	.	1	.	.	.
12	.	.	.	1	.	.	.	.	.	.	.	.	.	1
13	.	.	.	.	1	1	.	.	.	.	.	.	.	.
14	.	.	.	.	1	.	1	.	.	.	.	.	.	.
15	.	.	.	.	1	.	.	.	.	.	1	.	.	.
16	.	.	.	.	.	1	.	1	.	.	.	.	.	.
17	.	.	.	.	.	1	.	.	.	.	1	.	.	.
18	.	.	.	.	.	.	.	1	.	.	1	.	.	.
19	.	.	.	.	.	.	.	.	1	.	.	.	1	.
20	.	.	.	.	.	.	.	.	.	.	1	.	.	1

*Columns describe activity in seismic belts (1 = yes, 0 = no); rows are combinations of answers which form individual traits.

Kurile–Japan–Mariana belt and China are the two exceptions. G_1 characteristic traits occur with earthquake activity as a feature (as defined by the questions) in any one of the following seismic belts: central America, Peru–Chile, Tonga–Kermadec, New Guinea–New Hebrides and the Caribbean. Other G_1 traits require earthquakes in two or three belts. World seismicity does not show up by itself as a G_1 trait. These results imply a preferential excitation of the wobble by earthquakes in certain places (defined by latitude, longitude, fault and slip orientation)[1]. The surprising exception of the Japanese and Chinese region is a puzzle; perhaps slow deformation occurs in these belts during aseismic periods many years before or after major earthquakes. Perhaps some unknown compensatory mechanism is at work in these regions which counteracts the effect of earthquakes elsewhere. It seems that the overall pattern indicates that the excitation of the Chandler Wobble is related to earthquakes in specific places. The effect of seismic activity extends over several years before and after (mostly before) the year being tested; an expected result since the Chandler Wobble data were smoothed by taking 6-yr running averages.

The pattern recognition run for all 22 questions produced the characteristic traits shown in Figs 1 and 2. The display in

Table 3 Characteristic traits for G_2 years

Trait	Question													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	.	.	1	.	.	0	.	.	.	.	.	.	0	.
2	.	.	.	1	0	.	.	.	.	.	.	.	.	.
3	.	.	.	1	.	0	.	.	.	.	.	.	.	.
4	.	.	.	1	.	.	.	.	.	.	0	.	.	.
5	.	.	.	.	0	.	.	.	.	.	.	0	.	.
6	.	.	.	.	0	.	.	.	.	.	.	.	0	.
7	.	.	.	.	.	0	0	.	.	.	.	.	0	.
8	.	.	.	.	.	0	.	.	.	.	0	0	.	.
9	.	.	.	.	.	0	.	.	.	.	.	0	.	0
10	.	.	.	.	.	0	.	.	.	.	.	.	0	0
11	.	.	.	.	.	.	.	0	.	.	.	0	.	0
12	.	.	.	.	.	.	.	.	.	.	.	0	.	0

Fig. 1 Display of 8 G_1 traits associated with years in which amplitudes of the Chandler Wobble are increasing. The component features of each trait are shown in boxes in the proper time sequence with respect to a G_1 year. The number of the question (Table 1) from which the feature is derived is given in each box as an aid to the abbreviations. Eq, Earthquake; l.o.d., length of day.

these figures is organised to show both the time relationships and the way in which questions combine to form traits. Each trait is shown in a vertical column as a triplet or doublet of answers to questions; no singlets emerged as characteristic traits in this study. The vertical position of an answer reflects the time lag or advance in years with respect to the year being tested, which gave the best separation between G_1 and G_2 years in the preliminary one-dimensional survey mentioned earlier.

A dominant feature which emerges for G_1 years is the small value of rotational acceleration (characteristic traits 1, 5, 7, 8). The Earth does not seem to undergo accelerations or decelerations in its rotational velocity, ω, during years of increasing amplitude in the Chandler Wobble and associated increases of seismicity. Trait 7 implies a drop in the rotational acceleration during the years before a G_1 year, to the small values of $\dot{\omega}/\omega$ that go with such a year. This suggests a transition from an earlier G_2 cycle when $\dot{\omega}/\omega$ is large. Association with seismicity occurs, as discussed here, although fewer seismic belts are involved because features not involving earthquakes are now available to make up the quota of traits.

The characteristic traits for the G_2 years of decaying Chandler Wobble amplitudes show an absence of seismicity (traits 1–6). The Earth's rotational acceleration is large in G_2 years (traits 3, 4, 6, 7). Trait 7 identifies a period of volcanic activity ending two years before G_2 years with large values of rotational acceleration. Perhaps the climatic changes induced by volcanic

dust lead to zonal wind changes which perturb the rotational velocity[7]; however, this trait occurs in association with G_2 years when seismicity is low in 11 out of 13 earthquake belts. G_2 traits 4, 6, and 8 describe a pattern in which a large change in rotational velocity occurs near a time when the westward drift is slow; this is followed by a speed-up in the westward drift of the eccentric geomagnetic dipole 3–5 yr later. Therefore, the fast but slowing drift associated with G_1 years (G_1 traits 2, 3, 4 and 6) may be a relic of that acceleration in drift in a preceding G_2 period. The algorithm seems to have recognised the well known lag of several years between the changes in the length of the day and the motion of the eccentric dipole, which has generally been interpreted[5,6] as the time required for the magnetic fluctuation to diffuse through the mantle.

Hypothesis based on pattern recognition

Although we cannot prove that the traits individually or collectively carry significant information, the patterns that emerge invite speculation. In this vein we propose the following hypothesis. Seismic activity in particular belts is associated with displacements of lithospheric plates, which are sufficient to excite the Chandler Wobble. The displacements for these particular earthquakes include as a component large preseismic and postseismic deformation. In a subsequent seismically inactive period the Chandler Wobble decays by a transfer o.
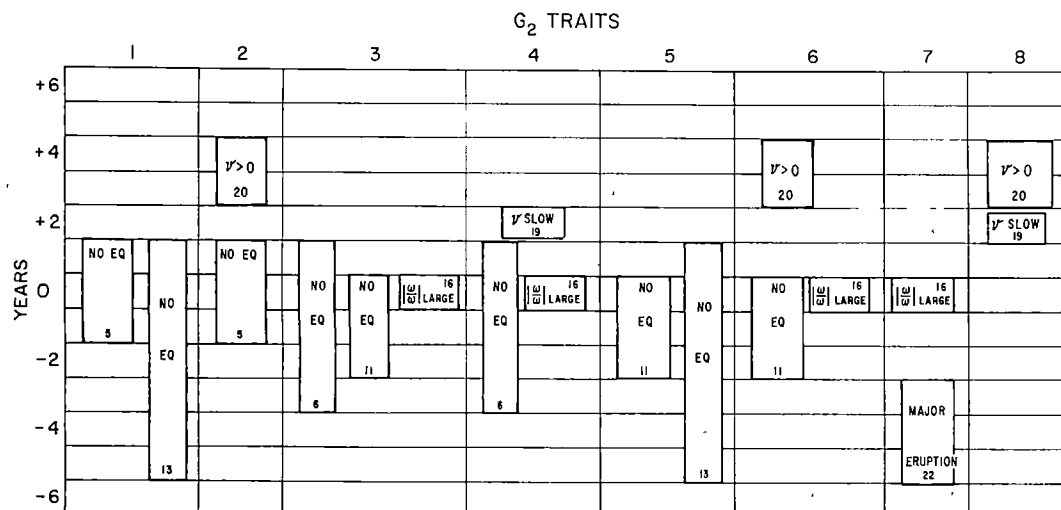
Fig. 2 Display of eight traits associated with years in which amplitudes of the Chandler Wobble are decreasing. Abbreviations as in Fig. 1.

angular momentum to the Earth's fluid core. The core motions produce compensatory changes in the rotation of the Earth, conserving angular momentum. The motions in the core also perturb the geomagnetic field, which becomes evident on the surface after several years of diffusion through the mantle.

The location of the energy sink for the Chandler Wobble is a subject of considerable speculation[13]. The notion that emerges from pattern recognition, that the wobble is damped by a transfer of its angular momentum to the core, should be analysed quantitatively for feasibility. Perhaps a non-spherical or bumpy core–mantle interface facilitates the transfer. Pattern recognition indicates that tectonic movements excite the wobble. The proposal that both may have a common cause cannot be ruled out, although its acceptance is unnecessary now that slow deformation and seismic slip in combination seem sufficient to excite the wobble.

This study was supported by the US Geological Survey, Department of the Interior. We are grateful to I. M. Gelfand, Sh. A. Guberman, and V. I. Keilis-Borok for making the

pattern recognition algorithm available to us. W. H. Press suggested that a spurious data test using time-reversed data was more convincing than one based on an answer matrix with random answers.

1 Dahlen, F. A., *Geophys. J.*, 32, 208–217 (1973).
2 Smylie, D. E , and Mansinha, L., *Geophys. J.*, 23, 329–354 (1971).
3 Kanamori, H., and Cipar, J. J , *Phys Earth planet. Interiors*, 9, 128–136 (1974).
4 Lambeck, K., and Cazenave, A., *Geophys., J.*, 32, 79–93 (1973).
5 Kahle, A B., Ball, R. H., and Cain, J C., *Nature*, 223, 165 (1969).
6 Vestine, E. H , and Kahle, A B., *Geophys. J.*, 15, 29–38 (1968).
7 Anderson, D. L., *Science*, 182, 49–50 (1974).
8 Bongard, M. M., Vaincvaig, M. H., Guberman, Sh. A., Izvekova, M. L., and Smirnov, M. S., *Geologiya Geofiz. Novosibirsk*, 6, 96–105 (1966).
9 Myerson, R. J , in *Earthquake Displacement Fields and the Rotation of the Earth*, (edit. by Mansinha, L., Smylie, D. E , and Beck, A), 159–168 (Springer, New York, 1970).
10 Duda, S., *Tectonophysics*, 2, 409–452 (1965)
11 Markowitz, W., in *Earthquake Displacement Fields and the Rotation of the Earth*, (edit. by Mansinha, L., Smylie, D. E., and Beck, A), 68–81 (Springer, New York, 1970).
12 Lamb, H., *Phil. Trans. R. Soc.*, A266, 425–534
13 Rochester, M. G., in *Earthquake Displacement Fields and the Rotation of the Earth*, (edit. by Mansinha, L., Smylie, D. E. and Beck, A.), 3–13 (Springer, New York, 1970).

A-Protein gene of bacteriophage MS2

W. Fiers, R. Contreras, F. Duerinck, G. Haegmean, J. Merregaert, W. Min Jou, A. Raeymakers, G. Volckaert & M. Ysebaert
Laboratory of Molecular Biology,

J. Van de Kerckhove, F. Nolf & M. Van Montagu
Laboratory of Histology and Genetics, State University of Ghent, Ledeganckstr. 35, B—9000 Ghent, Belgium.

The nucleotide sequence of the A-protein gene of bacteriophage MS2 has been determined and a model for its secondary structure is proposed. Also the amino acid sequence of the A-protein has been almost completely elucidated.

BACTERIOPHAGE MS2, and the closely related R17 and f2, contain three genes coding for the A-protein, the coat protein and the RNA polymerase subunit[1,2]. The first gene is located 5'-proximal[3]. The A-protein contains five histine residues, whereas the coat contains none[4]. It has a molecular weight of 38,000–42,000 and is present in one copy per virion[5–7]. A-protein is required for proper encapsidation as infection with an amber A-mutant in non-permissive conditions leads to production of non-infectious particles, which have a normal shell of coat protein, but from which part of the viral RNA 'dangles' out[8–10]. Selective removal of A-protein by high salt treatment of the virions correlates closely with loss of viability[11]. Although in a normal infection the A-protein enters the host cell together with the RNA and may have a role in the early cytoplasmic events[12,13], it is apparently not absolutely essential as free viral RNA is infectious in a spheroplast system[14].

In R17 the A-protein gene starts with A–U–G and the nucleotide sequence of the surrounding ribosome binding region has been elucidated[15]. We have shown that MS2 RNA starts with a 129-nucleotide long untranslatable leader sequence[16,17] and that the initiation codon of the A-protein gene is G–U–G (ref. 18). The termination signal is U–A–G and in su+am strains partial read-through occurs[7]. We have reported previously the last 135 nucleotides of the A-protein gene and have shown that the latter is followed by an intercistronic region 26 nucleotides long[19]. Some internal segments both of the

R17 and of the MS2 A-protein gene have also been published[20–24], as was the carboxyl-terminal amino acid sequence of the A-protein[25].

Now the complete nucleotide sequence of the A-protein gene has been determined. Also presented in this paper is a tentative model of the secondary structure. Moreover, in an independent effort the amino acid sequence of the MS2 A-protein itself has been almost completely elucidated.

Nucleotide sequence determination

By virtue of its specific secondary and tertiary structure highly ^{32}P-labelled MS2 RNA can be partially digested with RNase T_1 to yield discrete products[26,27]. Such a partial digest was first fractionated by electrophoresis on neutral polyacrylamide gel slabs and the resulting bands were further resolved into individual, pure fragments by two-dimensional gel electrophoresis[17,28]. These fragments were of chain length 25–250 and generally adequate for complete sequencing according to the methods developed by Sanger[29] or some modifications thereof[17,24,30]. Several fragments were often found to be related to each other (for example, one containing an extension of the other) and hence were derived from the same region of the RNA molecule; these related fragments are called a family. Many families could be identified by the presence of a unique T_1-oligonucleotide, like A–A–C–A–G or U–A–A–A–G (occurring once in MS2 RNA).

Some polypurine tracts have previously been located in the 5'-third of MS2 RNA[31] and on this basis some families could be assigned to the A-protein gene. Very mild T_1 digests, followed by separation on a 4% polyacrylamide gel, allowed the isolation of fragments in the size range of 200–1,000 nucleotides. In this
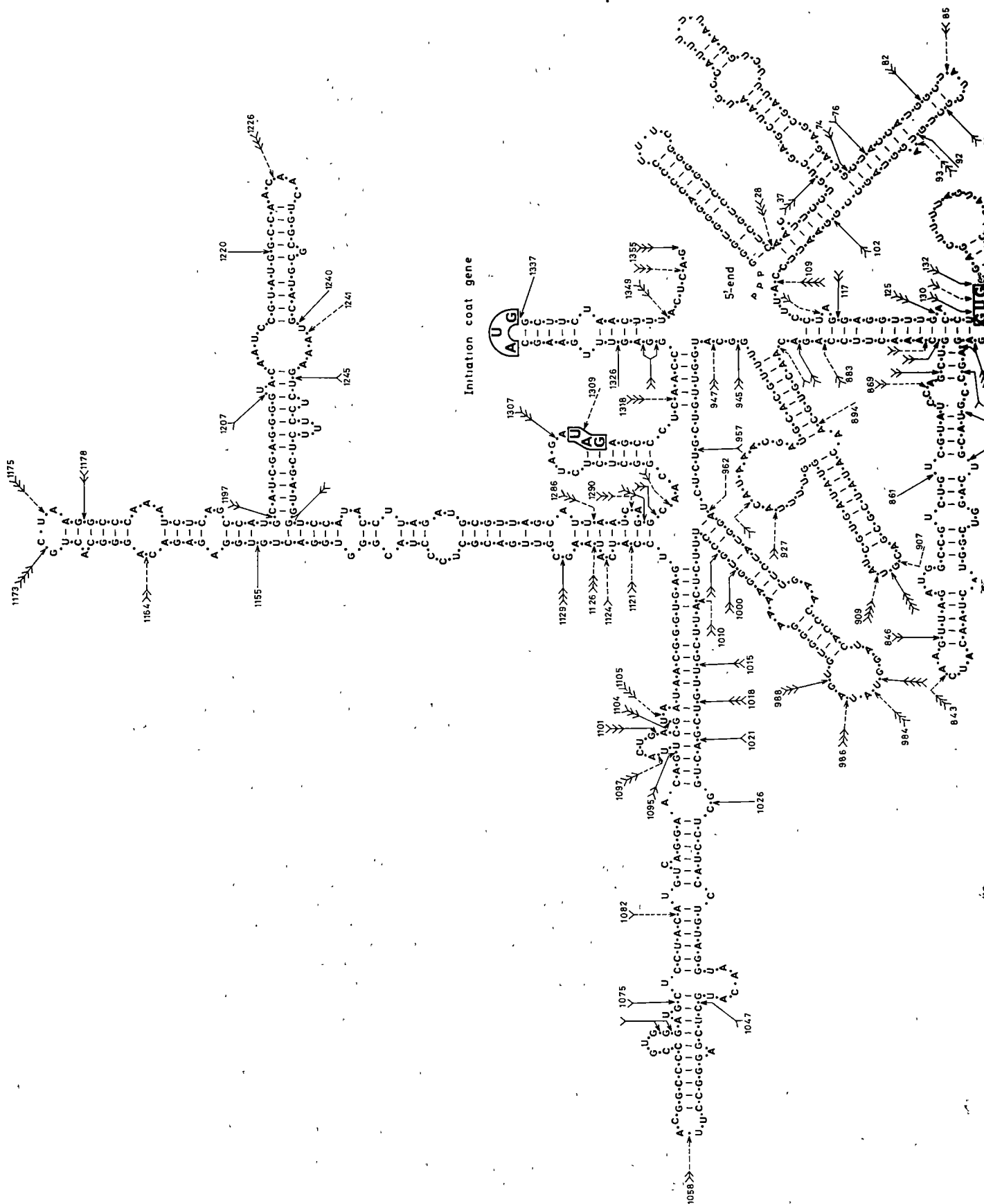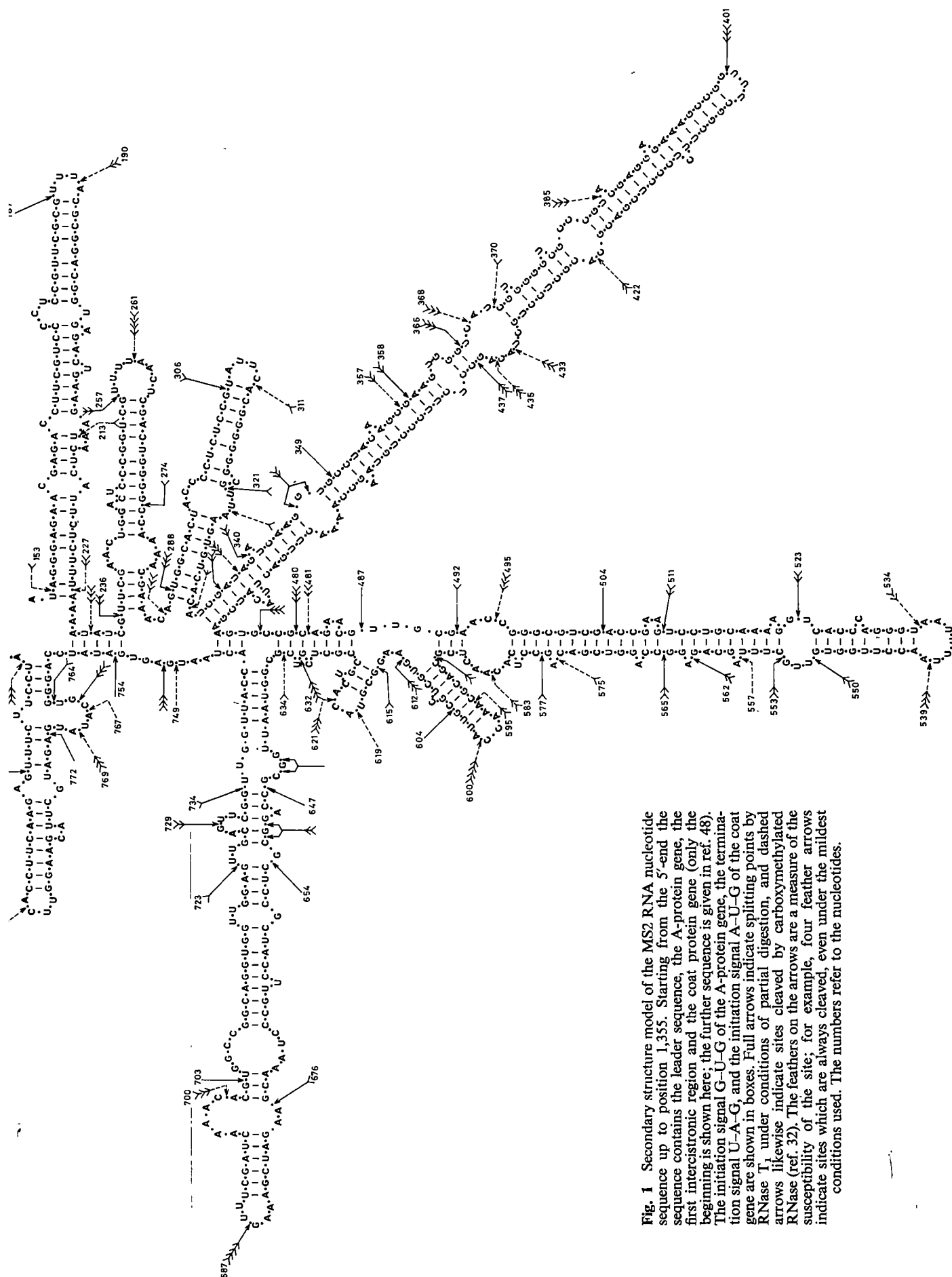
Fig. 1 Secondary structure model of the MS2 RNA nucleotide sequence up to position 1,355. Starting from the 5'-end the sequence contains the leader sequence, the A-protein gene, the first intercistronic region and the coat protein gene (only the beginning is shown here; the further sequence is given in ref. 48). The initiation signal G–U–G of the A-protein gene, the termination signal U–A–G, and the initiation signal A–U–G of the coat gene are shown in boxes. Full arrows indicate splitting points by RNase T₁ under conditions of partial digestion, and dashed arrows likewise indicate sites cleaved by carboxymethylated RNase (ref. 32). The feathers on the arrows are a measure of the susceptibility of the site; for example, four feather arrows indicate sites which are always cleaved, even under the mildest conditions used. The numbers refer to the nucleotides.

A-PROTEIN GENE

129
G-U-G C-G-A.G-C-U.U-U-U.A-G-U.A-C-U.C-U-C.G-A-U.A-G.G-A-G.A-A-C.G-A-G.A-C-C.U-U-C.G-U-C.C-C-C.U-C-C.G-U-U.C-G-C.G-U-U.
Met Arg Ala Phe Ser Thr Leu Asp Arg Glu Asn Glu Thr Phe Val Pro Ser Val Arg Val 20

189
U-A-C.G-C-G.G-A-C.G-G-U.G-A-G.A-C-U.G-A-A.G-A-U.A-A-C.U-C-A.U-U-U.C-U-C-U.U-U-A-A-A.U-A-U.C-G-U.U-C-G.A-A-C.U-G-G.A-C-U.
Tyr Ala Asp Gly Glu Thr Glu Asp Asn Ser Phe Ser Leu Lys Tyr Arg Ser Asn Trp Thr 40

249
C-C-C.G-G-U.C-G-U.U-U-U.A-A-C.U-C-G.A-C-U.G-G-G.G-C-C.A-A-A.A-C-G.A-A-A.C-A-G.U-G-G.C-A-C.U-A-C.C-C-C.U-C-U.C-C-G.U-A-U.
Pro Gly Arg Phe Asn Ser Thr Gly Ala Lys Thr Lys Gln Trp His Tyr Pro Ser Pro Tyr 60

309
U-C-A.C-G-G.G-G-G.G-C-G.U-U-A-A.G-U.G-U-C.A-C-A.U-C-G.A-U-A.G-A-U.C-A-A.G-U.G-C-C.U-A-C.A-A-G.C-G-A.A-G-U.G-G-G.U-C-A.
Ser Arg Gly Ala Leu Ser Val Thr Ser Ile Asp Gln Gly Ala Tyr Lys Arg Ser Gly Ser 80

369
U-C-G.U-G-G.G-G-U-C-G-C.C-C-C-G.U-A-C.G-A-G.G-A-A.A-A-G.C-C-G.G-U.U-U-C.G-G-C.U-U-C.U-C-C.C-U-C.G-A-C.G-C-C.U-C-C.
Ser Trp Gly Arg Pro Tyr Glu Glu Lys Ala Gly Phe Gly Phe Ser Leu Asp Ala Arg Ser 100

429
U-G-C.U-A-C.A-G-C.C-U-C.U-U-C.C-C-U.G-U-A-A.G-C.C-A-A.A-A-C.U-U-G.A-C-U.U-A-C.A-U-C.G-A-A.G-U-G.C-C-G.C-A-G.A-A-C.G-U-U.
Cys Tyr Ser Leu Phe Pro Val Ser Gln Asn Leu Thr Tyr Ile Glu Val Pro Gln Asn Val 120

489
G-C-G.A-A-C.C-G-G.G-C-G.U-C-G.A-C-C.G-A-A.G-U-C.C-U-G.C-A-A.A-A-G.G-U-C.A-C-C.C-A-G.G-G-U.A-A-U.U-U-U.A-A-C.C-U-U.G-G-U.
Ala Asn Arg Ala Ser Thr Glu Val Leu Gln Lys Val Thr Gln Gly Asn Phe Asn Leu Gly 140

549
G-U-U.G-C-U.U-U-A.G-C-A.G-A-G.G-C-C.A-G-G.U-C-G.A-C-A.G-C-C.U-C-A.C-A-A.C-U-C.G-C-G.A-C-G.C-A-A.A-C-C.A-U-U.G-C-G.C-U-C.
Val Ala Leu Ala Glu Ala Arg Ser Thr Ala Ser Gln Leu Ala Thr Gln Thr Ile Ala Leu 160

609
G-U-G.A-A-G.G-C-G.U-A-C.A-C-U.G-C-C.G-C-U.C-G-U.C-G-C.G-G-U.A-A-U.U-G-G.C-G-C.C-A-G.G-C-G.C-U-C.C-G-C.U-A-C.C-U-U.G-C-C.
Val Lys Ala Tyr Thr Ala Ala Arg Arg Gly Asn Trp Arg Gln Ala Leu Arg Tyr Leu Ala 180

669
C-U-A.A-A-C.G-A-A.G-A-U.C-G-A.A-A-G.U-U-U.C-G-A.U-C-A.A-A-A.C-A-C.G-U-G.G-C-C.G-G-C.A-G-G.U-G-G.U-U-G.G-A-G.U-U-G.C-A-G.
Leu Asn Glu Asp Arg Lys Phe Arg Ser Lys His Val Ala Gly Arg Trp Leu Glu Leu Gln 200

729
U-U-C.G-G-U.U-G-G.U-U-A.C-C-A.C-U-A.A-U-G.A-G-U.G-A-U.A-U-C.C-A-G.G-G-U.G-C-A.U-A-U.G-A-G.A-U-G.C-U-U.A-C-G.A-A-G.G-U-U.
Phe Gly Trp Leu Pro Leu Met Ser Asp Ile Gln Gly Ala Tyr Glu Met Leu Thr Lys Val 220

789
C-A-C.C-U-U.C-A-A.G-A-G.U-U-U.C-U-U.C-C-U.A-U-G.A-G-G.G-C-C.G-U-A.C-G-U.C-A-G.G-U-C.G-G-U.A-C-U.A-A-C.A-U-C.A-A-G.U-U-A.
His Leu Gln Glu Phe Leu Pro Met Arg Ala Val Arg Gln Val Gly Thr Asn Ile Lys Leu 240

849
G-A-U.G-G-C.C-G-U.C-U-G.U-C-G.U-A-U.C-C-A.G-C-U.G-C-A.A-A-C.U-U-C.C-A-G.A-C-A.A-C-G.U-G-C.A-A-C.A-U-A.U-C-G.C-G-A.C-G-U.
Asp Gly Arg Leu Ser Tyr Pro Ala Ala Asn Phe Gln Thr Thr Cys Asn Ile Ser Arg Arg 260

909
A-U-C.G-U-G.A-U-A.U-G-G.U-U-U.U-A-C.A-U-A.A-A-C.G-A-U.G-C-A.C-G-U.U-U-G.G-C-A.U-G-G.U-U-G.U-C-G.U-C-U.C-U-A.G-G-U.A-U-C.
Ile Val Ile Trp Phe Tyr Ile Asn Asp Ala Arg Leu Ala Trp Leu Ser Ser Leu Gly Ile 280

969
U-U-G.A-A-C.C-C-A.C-U-A.G-G-U.A-U-A.G-U-G.U-G-G.G-A-A.A-A-G.G-U-G.C-C-U.U-U-C.U-C-A.U-U-C.G-U-U.G-U-C.G-A-C.U-G-G.C-U-C.
Leu Asn Pro Leu Gly Ile Val Trp Glu Lys Val Pro Phe Ser Phe Val Val Asp Trp Leu 300

1,029
C-U-A.C-C-U.G-U-A.G-G-U.A-A-C.A-U-G.C-U-C.G-A-A-G.G-G-C.C-U-U.A-C-G.G-C-C.C-C-C.G-U-G.G-G-A.U-G-C.U-C-C.U-A-C.A-U-G.U-C-A.

Leu Pro Val Gly Asn Met Leu Glu Gly Leu Thr Ala Pro Val Gly Cys Ser Tyr Met Ser 320

1,089
G-G-A.A-C-A.G-U-U.A-C-U.G-A-C.G-U-A.A-U-A.A-C-G.G-G-U.G-A-G.U-C-C.A-U-C.A-U-A.A-G-C.G-U-U.G-A-C.G-C-U.C-C-C.U-A-C.G-G-G.

Gly Thr Val Thr Asp Val Ile Thr Gly Glu Ser Ile Ile Ser Val Asp Ala Pro Tyr Gly 340

1,149
U-G-G.A-C-U.G-U-G.G-A-G.A-G-A.C-A-G.G-G-C.A-C-U.G-C-U.A-A-G.G-C-C.C-A-A.A-U-C.U-C-A.G-C-C.A-U-G.C-A-U.C-G-A.G-G-G.G-U-A.

Trp Thr Val Glu Arg Gln Gly Thr Ala Lys Ala Gln Ile Ser Ala Met His Arg Gly Val 360

1,209
C-A-A.U-C-C.G-U-A.U-G-G.C-C-A.A-C-A.A-C-U.G-G-C.G-C-G.U-A-C.G-U-A.A-A-G.U-C-U.C-C-U.U-U-C.U-C-G.A-U-G.G-U-C.C-A-U.A-C-C.

Gln Ser Val Trp Pro Thr Thr Gly Ala Tyr Val Lys Ser Pro Phe Ser Met Val His Thr 380

1,269
U-U-A.G-A-U.G-C-G.U-U-A.G-C-A.U-U-A.A-U-C.A-G-G.C-A-A.C-G-G.C-U-C.U-C-U.A-G-A U-A-G

Leu Asp Ala Leu Ala Leu Ile Arg Gln Arg Leu Ser Arg 393

Fig. 2 Nucleotide sequence and amino acid sequence of the A-protein gene. Full lines indicate chymotrypsin peptides, dashed lines trypsin peptides and pointed lines thermolysin peptides; all these were completely sequenced. Also indicated are the out-of-phase termination signals. The number of nucleotides (from the 5'-end) are shown at the left and the number of amino acids at the right.

way all families belonging to the A-protein gene could be identified and roughly ordered. Unambiguous overlaps, however, were established by partial digestion of MS2 RNA with carboxymethylated (CM) ribonuclease[32]; this enzyme cleaves mainly C–A and U–A bonds. The CM partial digests of total MS2 RNA were fractionated in the same way as the T_1 digests. The resulting CM fragments not only provided all the required overlaps, but also allowed to sequence some regions which were represented in poor yield in the set of T_1 fragments.

For example the T_1 fragments 133–366 (positions are given in nucleotide units) and 367–632 were characterised. The former could be linked to the 5'-end[16-18] by virtue of the CM fragment 110–261, which a.o. contains the unique reference oligonucleotide A–G–G–A–G–G–U. Both T_1 fragments could then be linked by the CM fragment 339–433. Another CM fragment, 601–700 overlapped the second and the third T_1 family and so on. The detailed derivation of the nucleotide sequence will be reported elsewhere.

The nucleotide sequence of the A-protein gene, together with the preceding 5'-leader sequence and the succeeding link to the coat protein gene is presented in Fig. 1 in the form of a secondary structure model. The latter is partly based on experimental evidence, such as segments which fractionate together unless denaturing conditions are used, and susceptibility of sites to cleavage by the largely single-strand specific T_1 or CM RNases. Furthermore the thermodynamic stability of alternative configurations can be estimated[33] and on this basis the stability of the secondary structure model can be maximised. These estimates, however, involve many uncertainties. Moreover, the structure is certainly further moulded by tertiary interactions, as have been identified in tRNA (ref. 34). For these reasons the model shown in Fig. 1 should be regarded as tentative.

Amino acid sequence

Pure MS2 A-protein was obtained by isolation of the A-protein–RNA complex according to Osborn *et al.*[35], phenolisation, chromatography on a Sephadex G-200 column in the presence of 1% sodium dodecyl sulphate (SDS) and further separation by preparative polyacrylamide electrophoresis in the presence

of SDS. Starting from 5 g of virus 25 mg A-protein was obtained. The carboxymethylated A-protein was digested with appropriate proteolytic enzymes. The soluble peptides were fractionated by gel filtration on Sephadex G-25 in 5% formic acid followed by a three-dimensional combination of electrophoresis and paper chromatography[36]. Peptides were detected with fluorescamine and characterised by the micro dansyl-Edman procedure[37]. Several "insoluble" peptides were labelled with fluorescamine[38] and purified by electrophoresis in the presence of SDS on 17.5% polyacrylamide gel. The results of the amino acid sequence determination are summarised in Fig. 2. Further methodological details will be published elsewhere. Note that the first seven amino acids and the last three were determined on the complete polypeptide.

Except for a few gaps and missing overlaps, the amino acid sequence can be reconstructed without reference to the nucleotide sequence. It is in total agreement with the amino acid sequence derived from the nucleotide sequence on the basis of the known universal genetic code. Occasional apparent discrepancies were encountered, for example a threonine in position 245. But analysis of the same virus stock as used for nucleotide sequence analysis revealed the presence of serine at this position.

Biological implications

As previously discussed[18], the MS2 A-protein gene starts with G–U–G. Although more than 10 ribosome binding regions have been identified so far in phage and bacterial messengers[2,39] all other normal initiation codons are A–U–G (G–U–G, however, seems to be a functional start codon in translational reinitiation[40]). The initiation site of the R17 A-protein gene contains also A–U–G (ref. 15), in spite of the fact that the sequence of the region is otherwise identical to that of MS2 and that the level of gene expression is approximately the same in the two phages. The starting methionine residue is not removed after biosynthesis; the same is true for the analogous sequence Met–Arg–Val ... of the UDP galactose 4-epimerase[41]. The first eight amino acids of R17 have previously been determined[42]; they are identical except for a Thr→Ala change.

The code words used in the A-protein message are summarised in Fig. 3. Except for U–G–U (which does occur in the coat protein gene) all sense codons are found in the A-protein gene. It is quite possible that protein synthesis is also regulated at the level of chain elongation rate and this could be due to modulating codons which would locally brake the speed of the ribosomes. Therefore the latter type of codons would be avoided in a gene expressed at high frequency such as the coat protein gene. Candidates for such a modulating function are a.o. A–U–A, A–G–A and A–G–G. One may also note that for

Fig. 3 Code words used in the A-protein gene. The frequency of occurrence of each code word is given. ⊙, Initiation codon; ○, termination codon. Numbers encircled indicate codons not used in the coat gene; note that the coat protein does not contain histidine.

some amino acids (Ile, Asn, Gly) one degenerate codon is much preferred over the other (Fig. 3). The secondary folding of the viral RNA is certainly very important both from a functional and from a structural point of view. Yet it is not evident whether degeneracy of the code plays a role in this respect; in the model of the A-protein gene (Fig. 1), 67.4% of all the nucleotides are involved in secondary interactions, while for third letters this amounts to 68.4%, that is only slightly higher. The occurrence of termination signals in the two illegitimate reading frames (Fig. 2) is about as expected from a random distribution.

The ribosome binding region of the A-protein gene is only accessible after partial degradation and loss of three-dimensional folding of the viral RNA (ref. 43). It has been proposed that this gene is expressed on chains *statu nascendi* and turned off on subsequent folding of the viral RNA[2,44,45]. We have experimental evidence which supports this hypothesis. This model of auto-control at a translational level can be explained on the basis of the secondary structure (Fig. 1). When nearly two-thirds of the A-gene has been synthesised, a region appears (874–885) which interacts with part of the ribosome binding region and in this way closes off access to the latter. Further information is required, however, to prove this structure–function relationship.

Sixty to seventy per cent of all MS2 amber mutants are affected in the A-protein gene[46]. There are 18 glutamine residues in the A-protein, of which nine are coded by C–A–G (most amber mutations are C–A–G→U–A–G transitions). Yet more than one-third of the A-protein amber mutations occur at a

single site, resulting in non-permissive conditions in a polypeptide 88% of full length[47]. This mutational hot spot corresponds to the C–A–G at position 1,164–1,166 (Fig. 1); obviously it is rather exposed and therefore easily accessible to mutagenic reagents. A similar conclusion has previously been made for the mutation-prone C–A–G codons in the coat gene[48].

Compared with the coat protein the A-protein has a higher leucine to isoleucine ratio (factor 2.6), a higher tryptophan content (factor 2.0) and in particular a higher arginine content (factor 2.4). The abundant arginine residues (for example, in regions 168–177 185–195 and 388–393) may account for the strong binding to the RNA. Indeed the interactions between the A-protein and the other components of the virion are salt sensitive[11]. Also some clustering of hydrophobic amino acids is observed (such as the regions 196–206, 261–267 and 272–303) and this may explain the general stickiness of the A-protein. It is conceivable that during the formation of the capsid a single coat protein molecule (out of the 180) is replaced by an A-protein molecule. There are no regions with extensive sequence homology in these two molecules however.

1 Fiers, W., in *Handbook of Genetics* (edit by King, R. C.), 1, 271–294 (1974).
2 Weissmann, C., Billeter, M A., Goodman, H. M., Hindley, J., and Weber, H., *A. Rev. Biochem.*, 42, 303–328 (1973).
3 Jeppesen, P. G. N., Argetsinger-Steitz, J, Gesteland, R. F., and Spahr, P F, *Nature*, 226, 230–237 (1970).
4 Argetsinger-Steitz, J., *J. molec Biol.*, 33, 937–945 (1968).
5 Nathans, D., Oeschger, M. P., Eggen, K., and Shimura, Y., *Proc. natn. Acad. Sci. U.S.A.*, 56, 1844–1851 (1966).
6 Argetsinger-Steitz, J , *J. molec Biol.*, 33, 923–936 (1968).
7 Remaut, E., and Fiers, W., *J. molec. Biol.*, 71, 243–261 (1972).
8 Lodish, H. F , Horiuchi, K., and Zinder, N. D., *Virology*, 27, 139–155 (1965).
9 Argetsinger, J. E , and Gussin, G. N., *J. molec. Biol.*, 21, 421–434 (1966).
10 Heisenberg, M., *Biochem. biophys Res. Commun.*, 27, 131–137 (1967).
11 Verbraeken, E., and Fiers, W., *FEBS Lett.*, 28, 89–92 (1972).
12 Kozak, M , and Nathans, D., *Nature*, 234, 209–211 (1971)
13 Krahn, P. M., O'Callaghan, R. J., and Paranchych, W , *Virology*, 47, 628–627 (1972).
14 Davis, J. E , Strauss, J. H., and Sinsheimer, R. L., *Science*, 134, 1427 (1961).
15 Argetsinger-Steitz, J., *Nature*, 224, 957–964 (1969)
16 De Wachter, R., Vandenberghe, A., Merregaert, J., Contreras, R., and Fiers, W., *Proc. natn. Acad Sci. U.S A*, 68, 585–589 (1971).
17 De Wachter, R , Merregaert, J., Vandenberghe, A., Contreras, R , and Fiers, W., *Eur J. Biochem.*, 22, 400–414 (1971).
18 Volckaert, G., and Fiers, W., *FEBS Lett.*, 35, 91–96 (1973).
19 Contreras, R , Ysebaert, M., Min Jou, W., and Fiers, W., *Nature new Biol.*, 241, 99–101 (1973).
20 Rensing, U. F. E., *Biochem J.*, 131, 593–604 (1973)
21 Rensing, U. F E., and Coulson, A., *Biochem J.*, 131, 605–610 (1973).
22 Rensing, U. F. E., and Schoenmakers, J. G. G., *Eur. J. Biochem.*, 33, 8–18 (1973).
23 Rensing, U. F. E , Coulson, A , and Schoenmakers, J. G. G., *Eur. J. Biochem.*, 41, 431–438 (1974)
24 Haegeman, G , and Fiers, W., *Eur. J. Biochem* , 36, 135–143 (1973).
25 Vandekerckhove, J., Nolf, F , and Van Montagu, M , *Nature new Biol.*, 241, 102 (1973).
26 Min Jou, W , Hindley, J , and Fiers, W , *Archs intern. Physiol. Biochim.*, 76, 194–195 (1968)
27 Adams, J. M., Jeppesen, P. G. N , Sanger, F., and Barrell, B G., *Nature*, 223, 1009–1014 (1969).
28 De Wachter, R., and Fiers, W , *Analyt. Biochem.*, 18, 351–374 (1967)
29 Sanger, F., and Brownlee, G., *Methods in Enzymology*, 12A (edit. by Colowick, S P , and Kaplan, N O.), 361–381 (Academic, New York and London, 1967).
30 Contreras, R., and Fiers, W., *Analyt. Biochem.* (in the press)
31 Min Jou, W , Fiers, W., Goodman, H., and Spahr, P , *J. molec Biol.*, 42, 143–146 (1969)
32 Contreras, R , and Fiers, W., *FEBS Lett.*, 16, 281–283 (1971).
33 Tinoco, I., Borer, P N , Dengler, B., Levine, M. D., Uhlenbeck, O. C , Crothers, D. M , and Gralla, J., *Nature new Biol* , 246, 40–41 (1973)
34 Robertus, J. D., Ladner, J E , Finch, J. T., Rhodes, D , Brown, R. S , Clark, B F. C., and Klug, A., *Nature*, 250, 546–551 (1974)
35 Osborn, M , Weiner, A. M , and Weber, K , *Eur. J. Biochem.*, 17, 63–67 (1970).
36 Milstein, C , *Biochem J.*, 110, 652–654 (1968).
37 Bruton, C J , and Hartley, B. S , *J. molec. Biol* , 52, 165–173 (1970)
38 Vandekerckhove, J., and Van Montagu, M., *Eur. J. Biochem* , 44, 279–288 (1974)
39 Maizels, N., *Nature*, 249, 647–649 (1974).
40 Files, J G., Weber, K , and Miller, J. H., *Proc. natn. Acad Sci. U.S.A.*, 71, 667–670 (1974).
41 Musso, R. E , De Crombrugghe, B , Pastan, I., Sklar, J., Yot, P , and Weissman, S , *Proc. natn. Acad Sci. U S A* , 71, 4940–4944 (1974)
42 Weiner, A. M , Platt, J., and Weber, K., *J biol. Chem.*, 247, 3242–3251 (1972).
43 Argetsinger-Steitz, J , *Proc natn Acad Sci U S.A* , 70, 2605–2609 (1973)
44 Robertson, H D., and Lodish, H. F., *Proc. natn. Acad. Sci U S A* , 67, 710–716 (1970).
45 Kozak, M , and Nathans, D , *Bact Rev.*, 36, 109–134 (1972)
46 Fiers, W , et al , *Cold Spring Harb Symp. quant. Biol.*, 34, 697–706 (1969)
47 Vandamme, E , Remaut, E , Van Montagu, M , and Fiers, W., *Molec. gen Genet* , 117, 219–228 (1972)
48 Min Jou, W., Haegeman, G., Ysebaert, M., and Fiers, W , *Nature*, 237, 82–88 (1972).

Late Miocene sediments and fossils from the Northern Kenya Rift Valley

M. Pickford

Department of Geology, Queen Mary College, University of London, UK

The Lukeino Formation occurs within a long sequence of Neogene sedimentary units that are soundly calibrated by K–Ar dates. It has yielded an abundant and varied fauna and flora, and contains a wealth of information on palaeoenvironments and sedimentary processes occurring during its deposition. Previously unpublished vertebrate and invertebrate assemblages, about 6.5 Myr old, are recorded here, and the stratigraphic and sedimentary framework of the formation is established.

THE Lukeino Formation was mapped during 1973 as part of the programme dealing with all the sedimentary units in the Baringo area, Kenya, based at Bedford College, London, under Professor W. W. Bishop[1]. This work has resulted in the elucidation of the geographic extent, stratigraphic succession, facies changes, palaeoenvironments and palaeogeomorphological setting of the basin. During the survey many vertebrate and invertebrate fossils were collected including the crown of a hominid molar. Mapping has also shown that fossils collected previously from the Kaperyon area actually came from the Lukeino Formation. The Lukeino Formation was examined cursorily by Bishop et al.[1] in 1971, and this paper, apart from recording numerous new faunal elements, discusses in detail the sediments themselves.

Lukeino Formation

The Lukeino Formation (Figs 1–3) is composed of up to 130 m of primary and reworked volcanic rock. The sediments were deposited in a basin floored by the Kabarnet Trachyte Formation[2] and are overlain by the Kaparaina Basalt Formation[3]. Three sills have intruded the formation.
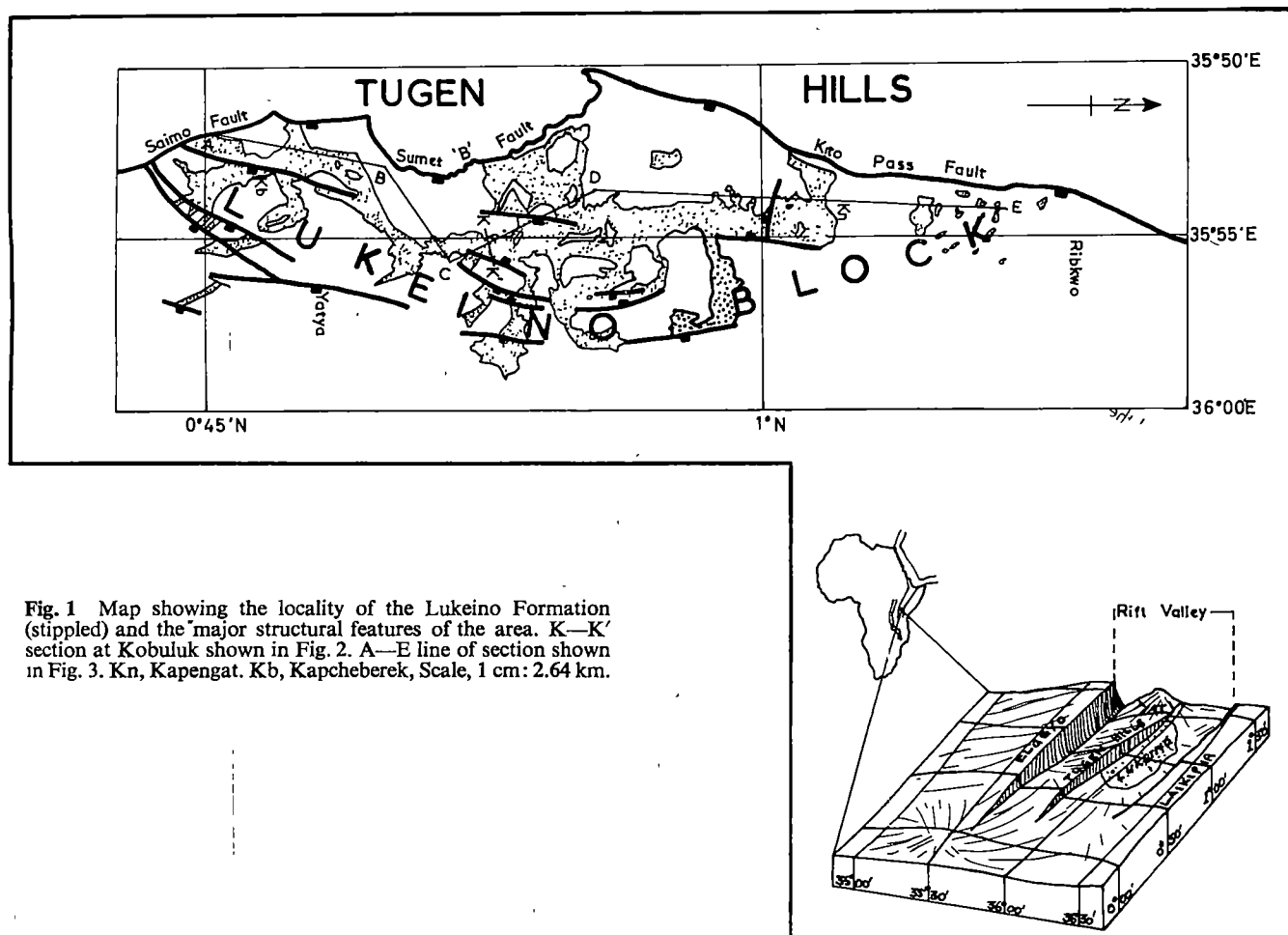
Fig. 1 Map showing the locality of the Lukeino Formation (stippled) and the major structural features of the area. K—K' section at Kobuluk shown in Fig. 2. A—E line of section shown in Fig. 3. Kn, Kapengat. Kb, Kapcheberek, Scale, 1 cm : 2.64 km.

Kaparaina Basalt

.54 ± 0.2Myr

─── unconformity ───

D

35m (115ft)

Fish and leaf impressions in shales

Diatomaceous and tuffaceous paper shales

Remobilised tuffaceous sediments

Kobuluk Sill

Porphyritic olivine dolerite.

C

12m (40ft)

Bedded grey pumice tuff with sandy and silty fractions

Few but varied freshwater molluscs

B

50m (168ft)

Diatomaceous and tuffaceous paper shales and silty tuffs with thin bands of grey tuff.

Palaeosols, suncracks and algal limestones.

Rormuch Sill

Porphyritic dolerite

B

Southern facies includes red beds (marls) fluviatile sands and tuffs, ferruginous horizons and palaeosols

Mammals common.

A

15m (50ft)

Red marls, tuffs, channel sands and discontinuous basal breccia

Numerous fossils including algae and bivalves.

─── unconformity ───

Kabarnet Trachyte Formation

6.7 ±,to 7.0 ±,Myr

LUKEINO FORMATION

KEY

algal limestone
fish impressions
gastropods
bivalves
mammals
shales
sands
silts
pumice tuffs
weak bedding
'intrusive tuff'
porphyritic lava and dolerite
palaeosol
channel
suncracks

Fig. 2   Type section of Lukeino Formation at Kobuluk.

The Lukeino unit is given formational status because it is clearly not part of the Kaparaina Basalt Formation in which it was originally included[1]. The maximum observed thickness occurs at Kobuluk (section K–K', Fig. 1) north of Yatya (0°52'30"N, 35°54'30"E) where 130 m of sediments are seen in an incomplete but continuous section. The base of the formation is not exposed in this area and the top was eroded before extrusion of the lowest local flow of Kaparaina Basalt. Extrapolation along strike suggests, however, that little is missing from the Kobuluk section. Poor exposure over much of the remaining outcrop of the Lukeino Formation has severely hampered stratigraphic study but the general relationships have been deciphered. Four members are recognised in the formation (A to D, Fig. 2).

A (15 m) is composed predominantly of red sediment but contains pale orange and yellow tuffaceous horizons. Occasional sand and grit-filled channels occur from which various fossils have been obtained including a hominid molar. Much of the epiclastic material is derived from ferruginous weathering products of the Kabarnet Trachyte. Varying amounts of pyroclastic material, possibly from contemporary volcanic sources as well as from the underlying Mpesida Beds[1], are mixed with the derived weathering products. The red beds seem to contain marginal lacustrine deposits as indicated by the presence of algal mats and lacustrine bivalves (including complete specimens with valves in the closed position).

The central and northern parts of B (50 m) consist of diatomaceous and pale cream silty tuffs, giving way southwards to red beds similar to those of A.

Well-bedded, grey, sandy and silty lapilli tuffs form a widespread Member C (12 m). They may be an extension northwards of the Riwo Beds of Martyn[4] which outcrop east of Kabarnet, about 35 km south-west of Yatya. The tuffs in the northern exposures contain a variety of lacustrine molluscs, while in the south they have yielded a few mammal bones. They are in general poorly fossiliferous.

Diatomaceous and silty tuffs comprise the major portion of Member D (35 m). Minor amounts of pumice tuff occur in the sequence except in the Kobuluk River, 2 km north of Kobuluk section, where a very thick succession of tuffs, agglomerates and breccias indicates proximity to a volcanic centre (Fig. 4). Away from the centre there are widespread fine-grained diatomaceous silts and tuffs which in places are exceptionally rich in fish impressions.

In the central parts of the basin, an extensive silty tuff outcrops in the diatomaceous sequence. The remarkable aspect of this unit is its 'intrusive' appearance. Its upper surface has many structures usually associated with igneous intrusions, such as cross cutting, stoping and distortion of bedding, but no baking was seen. This unit extends at least 12 km from north to south. Close examination shows that it is made up of small fragments of pre-existing sediments, sometimes well-laminated and it seems to represent a remobilised tuff. The intrusive nature of the contacts

possibly results from slumping of an unconsolidated stratum, accompanied by rupturing of overlying and underlying laminated diatomaceous tuffs.

Sills

Three sills have intruded the Lukeino Formation, two of which were emplaced before the deposition of Member D. The lowermost of these (The Rormuch Sill) is a coarse porphyritic dolerite with a fine-grained matrix. Honey-coloured feldspar phenocrysts up to 2 cm long are scattered throughout the sill, but become smaller northwards Intrusive contacts are displayed in several outcrops. The second sill (the Kobuluk Sill) is finer grained than the Rormuch Sill, and contains olivine phenocrysts up to 2 mm long set in a fine-grained grey matrix. The upper parts of this unit are often vesicular. The upper surface of this sill is often flow-banded and possesses pressure ridges, reminiscent of ropy lava, but its intrusive character is indicated in several outcrops. Fragments of both sills are found in the agglomerates of the Kobuluk River volcanic centre, which must therefore postdate the emplacement of the sills.

The third sill (the Cheseton Sill) has intruded sediments overlying tuffs and agglomerates of the Kobuluk River volcanic centre. It is very fine grained with occasional feldspar phenocrysts and indeed resembles a basalt flow. Its intrusive nature is indicated by a glassy upper surface, baked overlying sediments and the observation that it changes horizon.

Post-Lukeino times

Relative uplift of the Lukeino area after the deposition of Member D resulted in erosion of the formation, and in places valleys were cut through the sediments to the underlying Kabarnet Trachyte. The eroding landscape was eventually capped by basalts of the Kaparaina Formation, although in the north the basalts are now absent and the Lukeino Formation is overlain unconformably by sediments of the Chemeron Formation (Fig. 3 and ref. 5). The northern part of the Lukeino Block may not have been covered by Kaparaina Basalt, a suggestion prompted by thinning of the Basalts and a decrease in the number of flows northwards.

Palaeogeomorphology and palaeoenvironments

Fig 4 shows the palaeoenvironments and facies distribution in the Lukeino Formation. The Lukeino basin was initiated as a result of tectonic activity along the Kito Pass, Sumet 'B' and Saimo faults (Fig. 1) which formed the western boundary against which the sediments were ponded. The large mountain mass of Tiati volcano to the north was the probable northern bounding feature (Fig. 1). The Lukeino sediments are still relatively thick where they disappear under the Ribkwo Trachyte in the north of the area. To the south the sediments are obscured under an extensive pile of Kaparaina Basalt. To the east the sediments are downfaulted and covered by up to 280 m of Kaparaina

A   B   C   D   E

4000 ft

3000 ft

Alluvium
Ribkwo Trachyte
Chemeron Formation
Kaparaina Basalt
Lukeino Formation
Kabarnet Formation

0   5   10
km
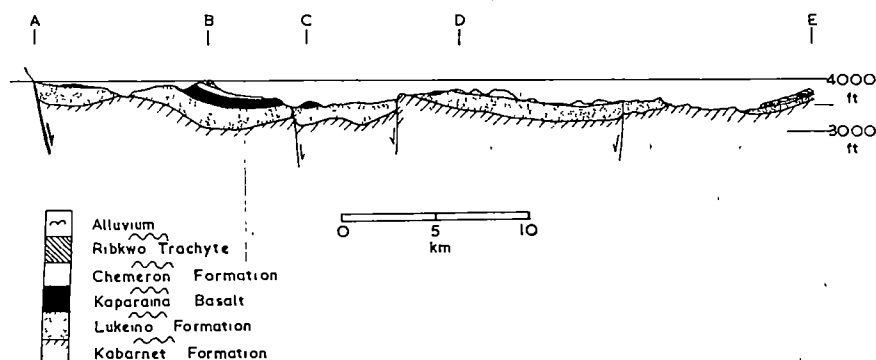
Fig. 3 North–south section through the Lukeino Formation. In the south Lukeino Formation sediment is overlain by Kaparaina Basalt, which in turn is overlain by Chemeron Formation sediment. In the north the Chemeron Formation oversteps the Lukeino Formation. A—E refer to localities indicated in Fig. 1.
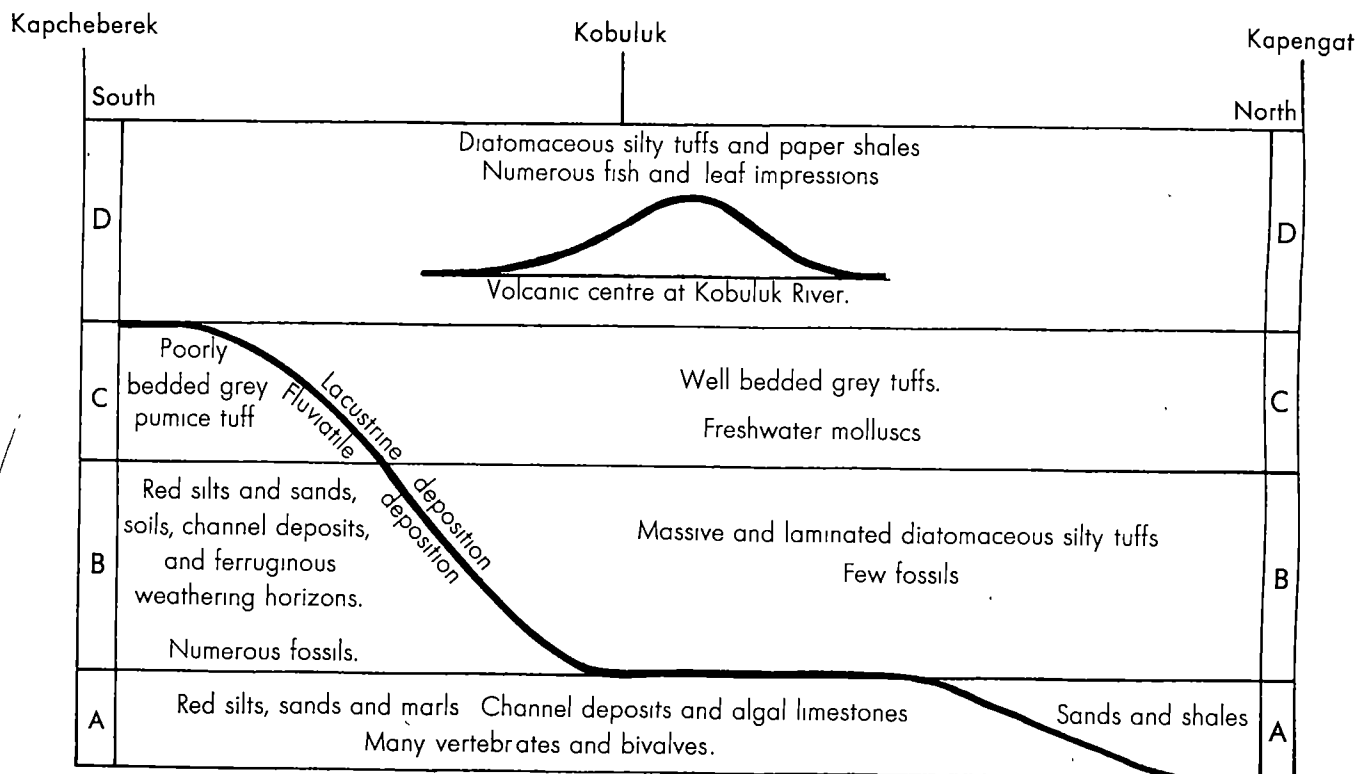
Fig. 4 Palaeoenvironments and facies distribution of the Lukeino Formation.

Basalt. The formation is exposed over an area of about 450 km² but outcrop is not continuous (Fig. 1). Sediments were derived from north, east and south, as well as from the growing Tugen Hills to the west and the Kobuluk River volcano within the basin. Much of the basal and lake margin sediment is red, and by analogy with present-day conditions, seems to be reworked from soils developed on the Kabarnet Trachyte. Algal limestones found in the red bed sequence suggest a lake marginal setting.

The grey tuffaceous sediments of Member C seem to be lacustrine in the north where they are well bedded and contain lacustrine gastropods and bivalves. In the south they are more massive, coarser and contain weathered horizons attest to emergence during Member D times, but the tuff.

Sediments of Member D were deposited in lacustrine conditions. The central parts of the basin contain abundant fish and plant remains in paper shales. Grass, leaves and diatoms (*Melosira* sp. and others) occur with cyprinids and cichlids. The fish are very abundant and scattered along bedding planes in the paper shales. Occasional suncracked horizons attest to emergence during Member D times, but these horizons are not close stratigraphically to the fish localities.

Fauna and flora

The list of fauna collected from the Lukeino Formation (including the original Kaperyon[1] collections) is shown in Table 1. Fossils were recovered from 47 localities, most of which occur in Members A and B. Fossil plants include leaves, wood, grass, ferns and diatoms. Algae are common, usually occurring as algal mats. Many of the specimens await detailed study, but several additions have been made to the existing list[1].

Hippopotamidae make up a large proportion of the artiodactyl assemblage while bovids, suids and giraffids are scarce. This is probably a reflection of the lacustrine and lake marginal nature of the sediments. The lake was probably always fresh or weakly saline as indicated by the

molluscs, diatoms, algae and fish. Crocodiles are common, and at least two species are present (the nile crocodile and a new species of broadnosed crocodile (Tchernov, personal communication)).

Maglio[8,9] has expressed some concern over the anomalous appearance of two species of Proboscidea collected from a locality mapped as Kaperyon Formation[1]. Remapping has now shown that the Kaperyon Formation is composed of three separate bodies. The Proboscidea referred to above come from sediments of the Lukeino Formation and are about 1.5 Myr older than originally thought. This is in keeping with the broad 'stage of evolution' age assigned to them by Maglio.

Age

The Lukeino Formation unconformably overlies the Kabarnet Trachyte Formation from which K–Ar dates of 7.2 ± 0.3, 6.8 ± 0.2 and 6.7 ± 0.3 Myr have been obtained[1]. They are uncomfortably overlain by the Kaparaina Basalt Formation from which a single date of 5.4 ± 0.2 Myr has been obtained[1]. The sediments were probably deposited about 6.5 Myr ago. Continued subsidence east of the Tugen Hills since deposition of the Ngorora Formation[10] (between 12 Myr and 9 Myr) resulted in several periods of sediment deposition[1]. The Lukeino episode covers a period about which little is known south of the Sahara, and its position in a well calibrated sequence enhances its importance.

Hominid molar

A lower molar (Fig. 5), clearly hominoid in structure, was found a few feet from outcrops of a 0.5 m thick, pink sandy and gritty horizon with pyrolusite and ferruginous staining and concretions. The horizon lies in the middle portion of Member A at Chepboit (locality 2/219) 5.5 km west of Yatya.

At the discovery site, fossils are concentrated in the coarse basal 10 cm in which ferruginous concretions and scarlet pebbles are common. The colour of the hominid molar closely resembles that of teeth found *in situ* in the

Table 1   Faunal list, Lukeino Formation

*Mollusca	
*Gastropoda	*Melanoides sp.
	*2 other spp.
*Bivalvia	*2 spp.
*Arthropoda	
*Ostracoda	*undet.
*Annelida	*indet.
Pisces	
*Clariidae	indet.
*Cichlidae	*Tilapia sp?
*Cyprinidae	*undet.
*Aves	
Reptilia	
Chelonia	
*Pelomedusidae	indet
Trionychidae	indet.
*Testudinidae	*indet.
*Squamata	
*Varanidae	*indet.
Crocodilia	
*Crocodilidae	*Crocodylus niloticus, Laurenti
	*Crocodylus sp.
Mammalia	
*Primates	
*Cercopithecoidea	*undet.
*Hominoidea	
*Hominidae	*undet.
*Carnivora	
*Felidae	*large sp.
	*small sp.
*Canidae	*undet.
*Viverridae	*cf. Ichneumia sp.
*Hyaenidae	*cf. Crocuta sp.
*Mustelidae	*Enhydriodon cf. iluecai Villalta and Crusafont.
Proboscidea	
Elephantidae	Stegotetrabelodon orbus Maglio
	Primelephas gomphotheroides Maglio
*Deinotheriidae	*Deinotherium cf. bozasi Arambourg
Perissodactyla	
Equidae	*Hipparion turkanense Hooijer and Maglio
	*Hipparion cf. sitifense Pomel
Rhinocerotidae	2 spp.
*Chalicotheriidae	*undet.
Artiodactyla	
Suidae	Nyanzachoerus tulotos Cooke and Ewer
Hippopotamidae	Hippopotamus sp.
	*Giraffa cf. jumae
	Reduncini indet.
	*Gazellini undet.
	Antilopini indet.
	* ?Neotragini indet.
	*Tragelaphini undet.
*Tubulidentata	
*Orycteropodidae	*Orycteropus sp. large
	*Orycteropus sp. small
Rodentia	
Hystricidae	indet.
*Other	*indet.

*Additions to previous list in ref. 1.

basal layer. Furthermore the tooth is slightly rolled and the roots have been dissolved away in a similar fashion to teeth found *in situ* in the grit. It is also slightly stained by pyrolusite and has a small pyrolusite concretion stuck to it. Dr P. Andrews (British Museum, Natural History) supplied the following description of the tooth.

"KNM LU335 is a left lower molar of an hominoid
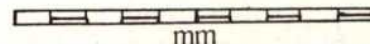
Fig. 5   KNM LU335, left lower molar, Hominidae indet.

primate. The crown was probably unerupted, and there is no sign of root development. The occlusal surface of the crown is intact but slightly pitted by weathering in places, and the base of the crown is slightly eroded so that nowhere is the basal limit present. The crown is short and broad : mesiodistal (md) length is 11.4 mm and buccolingual (bl) breadth 10.6 mm and the breadth/length index (bl/md × 100) is 93%. Talonid breadth and trigonid breadth are the same. The cusps are low and rounded, particularly the protoconid and hypoconid. The trigonid basin is small but quite deep. It is set well back from the low mesial marginal ridge of the crown and is bounded distally by a low transverse ridge connecting protoconid and metaconid. The talonid basin is small and crowded by the talonid cusps, so much so that the width of the basin is only half that of the total crown width. The length of the talonid basin is approximately equal to the lengths of the trigonid basin and distal fovea put together. The hypoconulid is placed buccal to the midline of the crown to a degree more often seen in M_2 than in M_1 in hominoid lower molars. The distal fovea is a deep pit distal to the low rounded hypoconulid-entoconid ridge which is cut by a very narrow groove connecting the distal fovea with the talonid basin. The groove system of the occlusal surface has a dryopithecine Y configuration. The buccal main groove and the buccal distal groove are particularly distinct, running between the buccal cusps to end in small pits on the remnants of the buccal cingulum, in the interval between cusps. Elsewhere the grooves are partly obscured by the secondary wrinkling which is most strongly developed in the talonid basin. Enamel thickness is very great.

Whether it is M_1 or M_2, KNM LU335 is considerably smaller than any of the australopithecines, *sensu lato*. It is closest in general dimensions to the M_2 of the chimpanzee. In morphology the Lukeino specimen is remarkably similar to the M_1 of robust australopithecines[6]. The most characteristic feature of the crown, and contributing to its breadth, is the lateral flare of its buccal edge. Since there are also faint traces of a buccal cingulum at the bases of the buccal grooves, it seems very likely that the lateral flare is formed as a result of incorporation of the cingulum into the occlusal surface of the crown, a process that in this tooth is not quite complete. The same feature is present in the hominoid upper molar from Ngorora[7] which is also low crowned, has a marked lingual flare, and has faint traces of a lingual cingulum.

Another feature of the Lukeino lower molar is the

approximately equal division of the crown into central (talonid) and mesial/distal basins. The protocone of the upper molar occludes with the talonid basin, and the hypocone with the basin constituted by the distal fovea of the equivalent lower molar and the trigonid basin of the lower molar next in line. The equal division of the lower molar crown into these two occlusal basins indicates that the upper molar had approximately equal sized protocone and hypocone. This is the case with the Ngorora upper molar, but is quite different from most dryopithecines, in which the lower molar trigonid basins occupy an insignificant proportion of crown length, the talonid basins occupying up to three quarters of the crown length, and the upper molar protocones are much larger than the hypocones. This pattern seen in dryopithecines is repeated in modern apes and also in *Ramapithecus wickeri* and some australopithecines, but in general hominids have more equally sub-divided lower molars, as in the Lukeino molar. KNM LU335, is thus tentatively included within the Hominidae."

I thank the Ministry of Natural Resources of the Government of Kenya for permission to study in Baringo District;

1 Bishop, W. W., Chapman, G. R., Hill, A. P., and Miller, J. A., *Nature*, 213, 389–394 (1971).
2 Walsh, J., *Rep. Geol. Surv. Kenya*, 84, 1–56 (1969).
3 King, B. C., and Chapman, G. R., *Phil. Trans. R. Soc.*, A271, 185–208 (1972).
4 Martyn, J. E., thesis, Univ. London (1969).
5 Martyn, J. E., *Nature* 215 (1967).
6 Robinson, J. T., *Transvaal Mus. mem.* 9, 1–179 (1956).
7 Bishop, W. W., and Chapman, G. R., *Nature*, 226, 914–918 (1970).
8 Maglio, V. J., *Nature* 225, 328–332 (1970).
9 Maglio, V. J., *Trans. Am. phil. Soc. N.S.*, 63 (3), 1–149 (1973).
10 Bishop, W. W., and Chapman, G. R., *Nature*, 226, 914–918 (1970).

Noble gases in lava rock from Mount Capulin, New Mexico

E. W. Hennecke & O. K. Manuel

Nuclear Division, Department of Chemistry, University of Missouri, Rolla, Missouri 65401

Noble gases in lava rocks from Mount Capulin crater cone contain 'parentless' ^{40}Ar and xenon trapped from the hot magma. The isotopic composition of the xenon is consistent with a mixture of 90% atmospheric and 10% solar xenon, but no radiogenic ^{129}Xe has been observed. These results do not support an earlier suggestion that radiogenic ^{129}Xe, found in CO_2 gas from this region of New Mexico, had been transported in hot magmas.

THE presence of a relatively large excess of radiogenic ^{129}Xe, the decay product of ^{129}I, has been recorded[1] in CO_2 well-gas from Harding County, New Mexico. The origin of the CO_2 gas is not understood, and the radiogenic ^{129}Xe may have been brought near to the Earth's surface in hot magmas[1,2], where intrusive contact with crustal carbonate rocks produced CO_2, during a phase of recent igneous activity in this region of New Mexico[3]. Reports on the iodine content of limestone[4] and the equilibrium ratio[5] of $^{129}I/^{127}I$ indicate however, that only a negligible fraction ($\approx 10^{-7}$) of the radiogenic ^{129}Xe

in the CO_2 gas could have come from the limestone.

We have already suggested[1] that lava rocks from this region of New Mexico might contain radiogenic ^{129}Xe, provided that xenon in the magma had not completely escaped as the lava cooled or had been diluted by exchange with atmospheric gases. Radiogenic ^{40}Ar can be used as a trace[6,7] to estimate the amount of gas loss from the hot lava, and a comparison of the abundances of trapped noble gases with those in the atmosphere[8,9] can be used to look for equilibration with atmospheric noble gases. We therefore undertook this study to measure the abundance and isotopic composition of the noble gases in a lava rock from this region of New Mexico, using a large specimen (≈ 2 kg) of lava rock from the Mount Capulin crater cone. The age of this cone has been estimated from ^{14}C dating to be between 4,400 and 10,000 years old[10].

The abundances and isotopic compositions were measured in two samples of the lava rock. To minimise surface contamination, the samples were taken from the interior of the specimen and were not crushed before gas extraction. They were mounted in the upper chamber of a water-cooled quartz extraction

Table 1 Noble gases released from lava rock, Mount Capulin

Sample	I	I	I	Blank I	I	II	II	Blank II	II
Weight (g)	18.164	18.164	18.164		18.164	15.865	15.865		15.865
Temperature (°C)	600	1,200	1,700	1,700	800–1,700	800	1,700	1,700	800–1,700
^{4}He	1.11±0.01	1.29±0.02	2.67±0.03	1.32±0.02	≈1.35±0.05	0.84±0.03	1.63±0.06	1.22±0.04	≈0.41±0.10
^{22}Ne*	0.013±0.001	0.58±0.02	1.59±0.05	0.022±0.001	2.16±0.07	0.33±0.01	2.24±0.02	0.015±0.001	2.56±0.03
^{36}Ar†	0.021±0.001	0.64±0.04	1.21±0.08	0.020±0.001	1.85±0.12	0.026±0.002	2.98±0.18	0.0064±0.0004	3.00±0.20
^{84}Kr†	0.031±0.001	0.40±0.01	0.95±0.03	0.017±0.001	1.35±0.04	0.25±0.07	1.96±0.53	0.0098±0.0026	2.20±0.60
^{130}Xe‡	0.48±0.02	0.47±0.02	1.63±0.08	0.15±0.01	2.43±0.13	0.37±0.02	3.34±0.20	0.21±0.01	3.50±0.23
^{40}Ar*		3.65±0.77	100.3±7.1		104±8	0.54±0.04	112±7		112±7

*Units of 10^{-9} cm^3 g^{-2}, at STP.
†Units of 10^{-11} cm^3 g^{-1}, at STP.
‡Units of 10^{-13} cm^3 g^{-1}, at STP.

Table 2 Isotopic compositions of noble gases released from Mount Capulin lava rocks

Sample	I	I	I	II	II	Air	Solar
Weight (g)	18.164	18.164	18.164	15.865	15.865		
Temperature (°C)	600	1,200	1,700	800	1,700		
^{20}Ne/^{22}Ne		9.77±0.03	9.87±0.03	9.80±0.04	9.84±0.03	9.81	13.0
^{21}Ne/^{22}Ne		0.0289±0.0001	0.0290±0.0001	0.0290±0.0001	0.0290±0.0001	0.0290	0.033
^{38}Ar/^{36}Ar		0.184±0.002	0.188±0.002	0.188±0.001	0.187±0.001	0.187	0.185
^{40}Ar/^{36}Ar		301.2±1.1	378.4±2.0	316.3±1.0	333.0±0.9	295.5	<0.6
^{78}Kr/^{84}Kr			0.0062±0.0001		0.0065±0.0002	0.0062	0.0064
^{80}Kr/^{84}Kr		0.0394±0.0005	0.0400±0.0003	0.0398±0.0002	0.0396±0.0005	0.0396	0.0401
^{82}Kr/^{84}Kr		0.200±0.001	0.202±0.001	0.201±0.001	0.201±0.001	0.202	0.202
^{83}Kr/^{84}Kr		0.200±0.001	0.202±0.001	0.201±0.001	0.201±0.001	0.202	0.202
^{86}Kr/^{84}Kr		0.306±0.001	0.304±0.001	0.305±0.001	0.305±0.001	0.305	0.304
^{124}Xe/^{130}Xe			0.0239±0.0004		0.0243±0.0003	0.0235	0.0273
^{126}Xe/^{130}Xe			0.0224±0.0003		0.0226±0.0002	0.0221	0.0261
^{128}Xe/^{130}Xe			0.478±0.005		0.483±0.004	0.470	0.506
^{129}Xe/^{130}Xe	6.45±0.03	6.45±0.03	6.49±0.03	6.51±0.04	6.47−0.02	6.48	6.32
^{131}Xe/^{130}Xe	5.17±0.06	5.16±0.03	5.17±0.02	5.20±0.05	5.16−0.03	5.19	5.01
^{132}Xe/^{130}Xe	6.57±0.05	6.60±0.03	6.55±0.01	6.59±0.05	6.53−0.03	6.59	6.13
^{134}Xe/^{130}Xe	2.56±0.01	2.55±0.02	2.52±0.01	2.45±0.04	2.54−0.02	2.56	2.29
^{136}Xe/^{130}Xe	2.18±0.01	2.17±0.01	2.14±0.01	2.16±0.01	2.16−0.02	2.17	1.87

The isotopic compositions of atmospheric Ne, Ar, Kr and Xe are from refs 33, 12, 34 and 35, respectively, and the isotopic compositions of solar Ne, Ar, Kr and Xe are from ref. 29.

bottle, the pressure was reduced to about 1×10^{-8} mmHg and the system blank was measured by collecting and analysing the gases generated by heating the empty molybdenum crucible to the highest extraction temperature, about 1,700 °C. After the system blank had been reduced to an acceptable level, the sample was dropped into the degassed crucible and the gases were extracted by stepwise heating. Extraction temperatures of 600 °C, 1,200 °C and 1,700 °C were used for sample I (18.164 g) and 800 °C and 1,700 °C were used for sample II (15.864 g). The samples were maintained at each temperature for 30 min. The gases released at each temperature were cleaned, separated and analysed using procedures described previously[11]. Standard volumes of air (≈ 0.01 cm^3 at standard temperature and pressure —STP) were analysed before and after the analysis of each sample to determine the sensitivity and mass discrimination of the mass spectrometer.

Noble gas abundances

The amounts of noble gases released at each extraction temperature are shown in Table 1, together with the uncertainties in the gas abundance measurements resulting from variations in the sensitivity of the mass spectrometer. The mass spectrometer signals for the 1,700 °C system blanks have been converted to effective gas contents (cm^3 g^{-1}, at STP). The isotopic ratios of the blanks were atmospheric to within ±5%, and blanks at 600, 800, and 1,200 °C were negligible except with ^{4}He when, at 600, 800 and 1,200 °C they were about 0.75 of their value at 1,700 °C. The relatively high blank level for He probably results from the diffusion of He into the glass vacuum system during the extraction and cleaning of gases. The total content of each noble gas, corrected for blanks, is shown in Table 2. In computing the totals we considered only the ^{4}He released at 1,700 °C, since the amounts released at lower temperatures were approximately equal to the ^{4}He blank level. The results of these analyses provide no information on the fraction of ^{4}He which is radiogenic, so we shall denote the concentration thus: (^{4}He).

The amounts of radiogenic ^{40}Ar (^{40r}Ar) were computed by assuming non-radiogenic argon to be of atmospheric composition[12] (Table 1). The average concentration of ^{40r}Ar is 108×10^{-9} cm^3 per gram of lava rock at STP. Decay of ^{40}K during the lifetime of the rock would generate only 28×10^{-9} cm^3 per g of potassium (STP). We interpret this as evidence of incomplete loss of ^{40r}Ar from the magma. These results suggest that radiogenic ^{129}Xe from the magma would also be trapped in the lava rocks, and its detection depends on the extent of dilution by atmospheric xenon.

To look for evidence of exchange of atmospheric noble gases with those in the lava rocks, we compared the abundance pattern of trapped noble gases in the lava rock with that in the atmosphere, using the method of Canalas et al.[13], who defined a fractionation factor, F^m, as

$$F^m = (^mX/^{130}Xe)_{sample}/(^mX/^{130}Xe)_{cosmic} \qquad (1)$$

where ^mX is any noble gas isotope of mass number, m, and cosmic abundances are those given by Suess and Urey[14].

The values of log F^m against mass number are shown in Fig. 1 for noble gases in the lava rocks (Table 1) and in the atmosphere[15]. Relative to the atmospheric abundances of noble gases, the average value of F^m in the lava rocks for ^{84}Kr is lower by a factor of 3, and for ^{22}Ne it is higher by a factor of 15. Within experimental uncertainties, the F^m values of ^{36}Ar and (^{4}He) are identical to those in the atmosphere. The irregular variation of F^m values with m (Fig. 1) does not fit the pattern that could be expected if equilibration with atmospheric gases is attained. Solubility equilibrium of atmospheric gases with the hot magma would have discriminated against the incorporation of large atoms[16], and adsorption equilibrium of atmospheric gases would have discriminated against small atoms[17]. The high Xe/Kr ratios in lava rock cannot be accounted for by the solution of atmospheric xenon in the parent magma, but may have resulted from adsorption.

Fig. 1 Log fractionation factor against mass number. The solid line shows the abundance pattern of noble gases in the atmosphere[15]; ○ noble gases in the Mount Capulin samples.
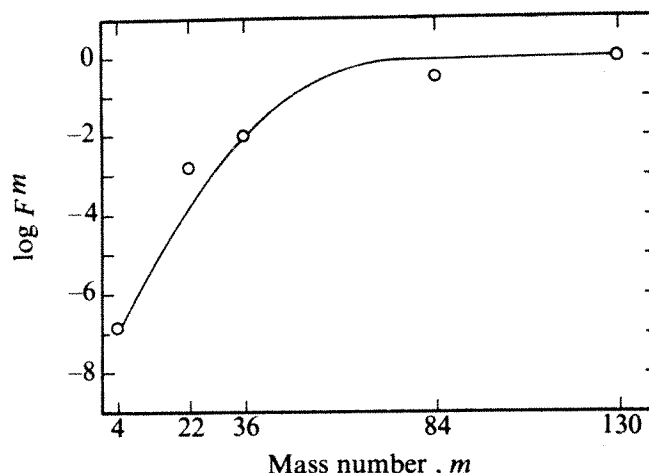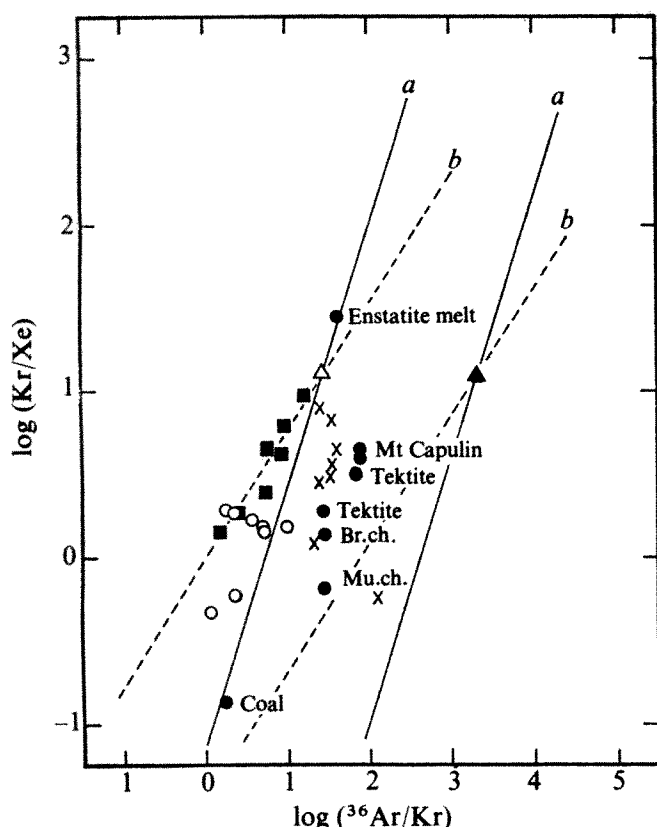
Fig. 2 A comparison of noble gas ratios in the Mount Capulin samples and in other material. *a*, Ratios expected in rocks which sample atmospheric and cosmic abundances of noble gases; *b*, slope used by Fisher[9,21] and Phinney[18]; ●, coal[13], Murray carbonaceous chrondrite (Mu. ch.)[26], Bruderheim chondrite (Br. ch.)[32], Thailand tektites[8], Mount Capulin lava rocks, and enstaitate melt[16]; ○, shales[13]; ■, basalts[9] from Pacific Seamount and Nigeria; ×, basalts from mid-ocean ridge reported to contain primordial noble gases[29-22]; △, air[15]; ▲, cosmic[14].

Stepwise extraction of gases is useful in separating gases from different sites[8,11], as adsorbed gases are preferentially released at the lower extraction temperatures, and the dissolved gases are enriched in gases released at higher extraction temperatures. Adsorption is highly specific for xenon[18], and the enrichment of xenon in the 600 °C fraction of sample I may result from the release of adsorbed gases. The bulks of all noble gases were, however, retained together with radiogenic ^{40}Ar until the samples were melted at 1,700 °C. As ^{40r}Ar was derived from the magma, the release of this isotope can be considered as a tracer for the release of noble gases incorporated into the rock from the magma. Although we cannot rule out completely the possibility of tenaciously adsorbed xenon being released in the 1,700 °C fraction, high Xe/Kr ratios can be expected in the magma as the results of other studies[13,17,18] indicate that the Xe/Kr ratio in air is anomalously low because of the preferential adsorption of xenon on fine-grained sediments. Since 'parentless' ^{40}Ar came in with the hot magma and is still present in the lava rock, there is an overwhelming probability that any radiogenic ^{129}Xe originally in the hot magma would also still be trapped in the lava rock.

The relatively high values of the Ne/Ar ratios in these lava rocks seem to confirm reports of a high Ne/Ar ratio in pumice[19] and of high Ne/Ar ratios in lava rocks from the sea floor[20]. Although the enrichment of lighter noble gases in the latter rocks has been interpreted as evidence of the presence of primordial rare gases from the Earth's mantle[20-22], it is possible that the enrichment results from the preferential leakage of lightweight atmospheric noble gases into rocks which have low concentrations of indigenous noble gases. Thailand tektites show a large excess of neon[8], but it has been shown,

from the diffusion coefficient of neon in tektite glass[23] and from the Kr–Ar[23,24] and fission track[25] age estimates of those tektites, that the high neon content could result from the diffusion of atmospheric neon into the tektite. The isotopic ratios of Ne and Xe in the atmosphere are quite different from those in meteorites[26] and from that imbedded in lunar fines[27].

Isotopic abundances

The isotopic composition of the noble gases released from Mount Capulin basalt are given in Table 2. No attempt has been made to measure the ^{3}He content, and there have been no corrections for blanks, because most of the isotopic ratios are essentially atmospheric. The isotopic compositions are atmospheric within $\pm 2\sigma$ ($\sigma = 1$ standard deviation), except for an obvious excess of radiogenic ^{40}Ar and perhaps a slight depletion of the heavy xenon isotopes in the 1,700 °C fraction of sample I and a slight enrichment of the light xenon isotopes in the 1,700 °C fraction of sample II.

We could not find radiogenic ^{129}Xe in lava rock from the region where radiogenic ^{129}Xe was found in CO_2 gas; however, the 'parentless' ^{40r}Ar and high Xe/Kr ratios in gases released at the highest extraction temperatures compel us to conclude that the Xe originated in the magma. The absence of radiogenic ^{129}Xe in these samples suggests that radiogenic ^{129}Xe *in the* CO_2 gas may come from some source other than the hot magma, and since it did not come from the decomposition of limestone[1], other origins should be considered. Leakage of CO_2 from an asthenosphere which contains a CO_2-rich liquid phase[28] seems to be a reasonable alternative which could account for both the high CO_2 content of the gas and the presence of the decay product of extinct ^{129}I in gas pockets trapped within the Earth's crust.

The isotopic ratios (Table 2) contain no evidence of solar-type noble gases, except for the slight enrichment of the light Xe isotopes and depletion of the heavy Xe isotopes in the 1,700 °C fractions. A mixture containing atmospheric Xe and solar Xe in the ratio 9:1, could account for the isotopic ratios in Xe released from both samples at 1,700 °C. Because of the very small differences between atmospheric Xe and that released from the Mount Capulin basalt at 1,700 °C, caution should be exercised in interpreting our results as evidence of primitive Xe within the Earth. The isotopic ratios of the lighter noble gases seem to be atmospheric.

Atmospheric or solar origin?

Previous observations cited as evidence that oceanic lava rocks contain primitive gases from the Earth's mantle include the similarity between the relative abundances of noble gases in some lava rocks and in meteorites[20-22], the strong contrast of these relative abundances with those in the atmosphere[20,21], the fact that the simple fractionation of atmospheric noble gases could not simultaneously produce higher values for the Ne/Xe ratio and lower values for the Kr/Xe ratio, and the failure of noble gas ratios in some lava rocks to lie along equilibrium correlation lines defined by Henry constants[16,30] for the solubility of atmospheric noble gases into a melt[20-22].

Fisher[9] first used the correlation of noble gas ratios for ^{36}Ar, Kr, and Xe on log–log plots to differentiate between noble gases from the atmosphere and noble gases from a solar (primordial) source. Subsequently, Dymond and Hogan[20] and Fisher[21] used these plots, and one involving Ne, to show that the noble gas contents in some deep sea lava rocks did not fit the equilibrium correlation line for atmospheric noble gases. For any four noble gases, a, b c, and d, the concentration ratios, C_a/C_b, C_c/C_d, and so on, in a melt at equilibrium with an atmosphere having partial pressures, p_a, p_b, p_c are defined by[9,16,30]

$$\ln(C_a/C_b) - \ln(p_a/p_b) =$$

$$[(r_a^2 - r_b^2)/(r_c^2 - r_d^2)] [\ln(C_c/C_d) - \ln(p_c/p_d)] \qquad (2)$$

where r_a is the atomic radius of the noble gas, a, and so on.

Figures 2 and 3 compare the noble gases in the Mount Capulin lava rocks with the equilibrium lines defined by equation (2) for noble gases from the Earth's atmosphere[15] and from an atmosphere containing cosmic abundances of noble gases[14]. Values of atomic radii given by Pauling[31] were used. The figures give the noble gas ratios used by Fisher[9,21,22] and by Dymond and Hogan[20] to distinguish primordial from atmospheric gases. Since the slope which we calculated from equation (2) for the plot of log(Kr/Xe) against log(^{36}Ar/Kr) is significantly steeper than that shown by Fisher[9,21] and Phinney[18], and since differences in the slopes may lead to different conclusions about the origin of noble gases in certain samples, their value for the slope has been shown (Fig. 2). Experimental values[16] for the solubility of He, Ne, and Ar in enstatite melts, and extrapolated values[16] for the solubility of Kr and Xe, have been used to calculate the noble gas ratios in a melt which sampled gases from the atmosphere, and these ratios agree with the solid equilibration lines (Fig. 2a).
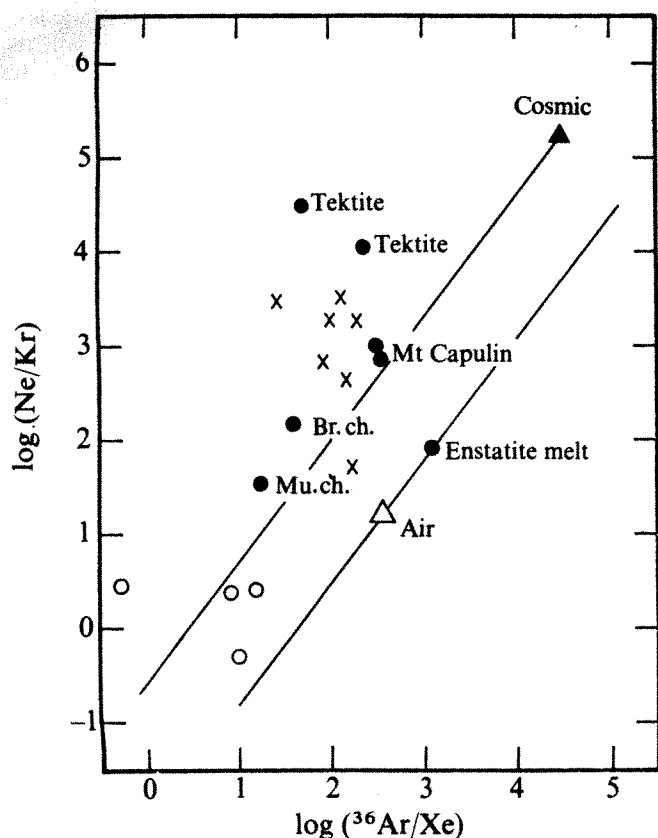
Fig. 3 A comparison of noble gas ratios in the Mount Capulin lava rocks and in other material. The solid lines were calculated from equation (2) and define the noble gas ratios in samples which have equilibrated with gases having atmospheric[15] and cosmic[14] abundances. Symbols as in Fig. 2.

All of the noble gas ratios in Fig. 2 represent terrestrial samples, except for two data points representing the Bruderheim chondrite[32] and the Murray carbonaceous chondrite[26]. The ratios of these three heavier noble gases in terrestrial samples lie on both sides of the atmospheric equilibration line, with the gases in three shale samples[13] and the coal sample[13] lying close to that expected from equilibration with atmospheric gases. Noble gases in seven basalt samples analysed by Fisher[9] are generally to the left of the atmospheric equilibration line, and noble gases in tektites[8], in samples from the Vema Fracture Zone of the Atlantic[21,22], in glassy outer portions of basalts

from mid-ocean ridge[20], and in the Mount Capulin basalts are generally to the right of the atmospheric equilibration line. One view of the data shown in Fig. 2 is that those terrestrial samples lying between the atmospheric and the cosmic equilibration lines may have sampled a mixture of atmospheric and primordial gases within the Earth[20-22].

All of the non-radiogenic noble gases, including Ne, have been included in Fig. 3. Fewer data are presented there because of the absence of Ne analyses on the coal, several shale samples, or basalts from the Vema Fracture Zone of the Atlantic. The noble gas ratios in the Mount Capulin basalts, as well as in the Bruderheim and the Murray chondrite, lie close to the ratios to be expected if equilibrium with cosmic noble gases had been obtained. All of the noble gas ratios in the terrestrial samples shown in Fig. 3, however, lie to the left of the air equilibration line, and many are shifted far to the left of the line which defines equilibration with cosmic abundances of noble gases. In view of the fact that the terrestrial samples, which lie to the left of the cosmic equilibration line in Fig. 3, lie between the atmospheric and cosmic equilibration lines in Fig. 2, it seems that the position of these noble gas ratios in Fig. 2 does not necessarily indicate equilibration of the melts with a mixture of primordial and atmospheric gases[20-22]. Rather, it seems that the low Kr/Xe ratios shown in Fig. 2 may result from the fact that the Kr/Xe ratio for the Earth is appreciably lower than the Kr/Xe ratio for the atmosphere, as has been noted earlier[13], and the high Ne/Kr ratios shown in Fig. 3 may result from the preferential leakage of Ne into igneous rocks which form near the Earth's surface and have very low amounts of indigenous noble gases.

These analyses reveal the presence of a large excess of 'parentless' ^{40}Ar, though radiogenic ^{129}Xe was not observed. The abundance pattern of gases released at the highest extraction temperatures suggests that there has been little or no exchange of atmospheric xenon with these gases. The absence of radiogenic ^{129}Xe in basalts which have trapped ^{40r}Ar from the magma implies that this is not the source of radiogenic ^{129}Xe in CO_2 gas wells.

1 Boulos, M. S., and Manuel, O. K., *Science*, 174, 1334 (1971).
2 Bulter, W. A., Jeffrey, P. M., Reynolds, J. H., and Wasserburg, G. J., *J. geophys. Res.*, 68, 3283 (1963).
3 Levorsen, A. I., *Geology of Petroleum* (edit. by Berry, F. A. F.), 220 (Freeman, San Francisco, 1967).
4 Becker, V. J. Bennett, J. H., and Manuel, O. K., *Chem. Geol.*, 9, 133 (1972).
5 Srinivasan, B., Alexander, E. C., Jr, and Manuel, O. K., *Science*, 173, 327 (1971).
6 Dalrymple, G. B., and Moore, J. G., *Science*, 161, 1132 (1968).
7 Funkhouser, J. G., Fisher D. E., and Bonatti, E., *Earth planet. Sci Lett.*, 5, 95 (1968).
8 Hennecke, E. W., Manuel, O. K., and Sabu, D. D., *J. geophys. Res.* (in the press).
9 Fisher, D. E., *Earth planet. Sci. Lett.*, 9, 331 (1970).
10 Muehlberger, W. R., *Bull. geol. Soc. Am.*, 66, 1600 (1955).
11 Hennecke, E. W., and Manuel, O. K., *Z. Naturf.*, 26a, 1980 (1971).
12 Nier, A. O. *Phys. Rev.*, 77, 789 (1950).
13 Canalas, R. A., Alexander, E. C., Jr, and Manuel, O. K., *J. geophys. Res.*, 73, 3331 (1968).
14 Suess, H. E., and Urey, H. C., *Rev. mod. Phys.*, 28, 48 (1956).
15 Verniani, F., *J. geophys. Res.*, 71, 385 (1966).
16 Kirsten, T., *J. geophys. Res.*, 73, 2807 (1968).
17 Fanale, F. P., and Cannon. W. A., *Earth planet. Sci. Lett.*, 11, 362 (1971).
18 Phinney, D., *Earth planet. Sci. Lett.*, 16, 413 (1972).
19 Lord Rayleigh, *Proc. R. Soc.*, A170, 451 (1939).
20 Dymond, J., and Hogan, L., *Earth planet. Sci. Lett.*, 20, 131 (1973).
21 Fisher, D. E., *Nature*, 244, 344 (1973).
22 Fisher, D. E., *Geophys. Res. Lett.*, 1, 161 (1974).
23 Reynolds, J. H., *Geochim. cosmochim. Acta*, 20, 101 (1960).
24 Zähringer, J., and Gentner. W., *Nature*, 199, 583 (1963).
25 Gentner, W., Storzer, D., and Wagner, G. A., *Geochim. cosmochim. Acta*, 33, 1075 (1969).
26 Reynolds, J. H., *Phys. Rev. Lett.*, 4, 351 (1960).
27 The Lunar Samples Preliminary Examination Team, *Science*, 165, 1211 (1969).
28 Green, H. W., III, *Nature*, 238, 1 (1972).
29 Srinivasan, B., Hennecke, E. W., Sinclair, D. E., and Manuel, O. K., Proc. third Lunar Science Conference, *Geochim. cosmochim. Acta*, suppl. 3(2), 1927 (1972).
30 Blander, M., Grimes, W. R., Smith, N. V., and Watson, G. M., *J. phys. Chem. Wash.*, 63, 1164 (1959).
31 Pauling, L., *The Nature of the Chemical Bond*, 514 (Cornell University Press, Ithaca, 1960).
32 Manuel, O. K., and Rowe, M. W., *Geochim. cosmochim. Acta.*, 28, 1999 (1964).
33 Eberhardt, P., Eugster, O., and Marti, K., *Z. Naturf.*, 20a, 623 (1965).
34 Nief, G., *Isotopic Abundance Ratios Given for Reference Samples Stocked by the National Bureau of Standards* (edit. by Mohler, F.), 51 (National Bureau of Standards, Washington, DC, 1960).
35 Nier, A. O., *Phys. Rev.*, 79, 450 (1950).

Existence and possible roles of transcriptional barriers in T7 DNA early region as shown by electron microscopy

Jean-Luc Darlix & Mireille Horaist

Service de Biochimie (142), Département de Biologie CEN Saclay, BP 2 91190 Gif/Yvette, France

Transcription of T7 DNA in vitro by Escherichia coli RNA polymerase in the absence or presence of termination factor p has been studied extensively using electron microscopy. It is demonstrated that sequences located between the early genes behave as transient transcriptional barriers in the absence of p and are recognised both as p-dependent terminators and as RNaseIII cleavage sites.

IN the current model for selective transcription, initiation and termination of RNA transcripts are dictated by specific signals on the DNA template that are recognised and translated into biochemical events by the transcription apparatus.

Specific initiation of RNA synthesis has been studied extensively *in vitro* using a model 'transcription of T7 DNA by *E. coli* RNA polymerase' that requires no added factor other than DNA and RNA polymerase holoenzyme[1]. It has been shown that a subunit of RNA polymerase holoenzyme, the σ factor, governs the tight binding of RNA polymerase molecules[1] to three specific sites within the early promotor region of T7 DNA (ref. 2), from which region RNA chain initiation takes place very rapidly in the presence of ribonucleoside triphosphates[3-5]. Darlix *et al.*[6] found that the subsequent elongation of RNA is discontinuous, that is, during the transcription of a long DNA sequence, regions of rapid transcription alternate with regions of slow transcription. More precisely, previous investigations have clearly indicated that the discontinuities in the transcription process are caused by sequences present only in natural DNAs and probably located between genes[7], and that such sequences in the T7 DNA early region could well include p-dependent termination sites[7].

We describe here the discontinuous propagation of *E. coli* RNA polymerase on T7 DNA template, as well as the action of p on this process, as observed by electron microscopy.

Discontinuous transcription of T7 DNA

Discontinuous transcription of DNA is best observed when the RNA chain growth rate is reduced; this is obtained by decreasing the concentration of one nucleotide[6]. As previously shown[7], transcription of T7 DNA with or without p is clearly discontinuous in conditions in which GTP concentration is lowered to 1.5×10^{-5} M. Successive plateaux occur during the kinetics of total RNA synthesis in the presence or absence of p. We have located the growing RNA on the physical map of T7 DNA at times corresponding to the plateaux (Fig. 1).

The DNA–RNA polymerase–RNA transcription complexes have been studied in the electron microscope by dark-field observation of specimens prepared by adhesion on positively charged carbon films[8]. In the present conditions and in the absence of p, growing RNA chains ap-

pear as clustered bushes on the DNA (Fig. 2). If transcription is stopped after 4, 11 and 25 min, that is, when RNA synthesis is particularly low (plateaus, Fig. 1), most active enzymes are found at or near the end of gene 0.3— the first one[6]—(Fig. 3a), the end of genes 0.3 and 0.7 (Fig. 3b), and the end of genes 0.7, 1 and 1.1 (Fig. 3c), respectively. Previous results of the RNA chain length analysis of the corresponding transcription products agree with the present findings[7]. In fact, the mRNA of gene 0.3, of genes 0.3 and 0.7, and of genes 0.3, 0.7 and eventually 1 and 1.1
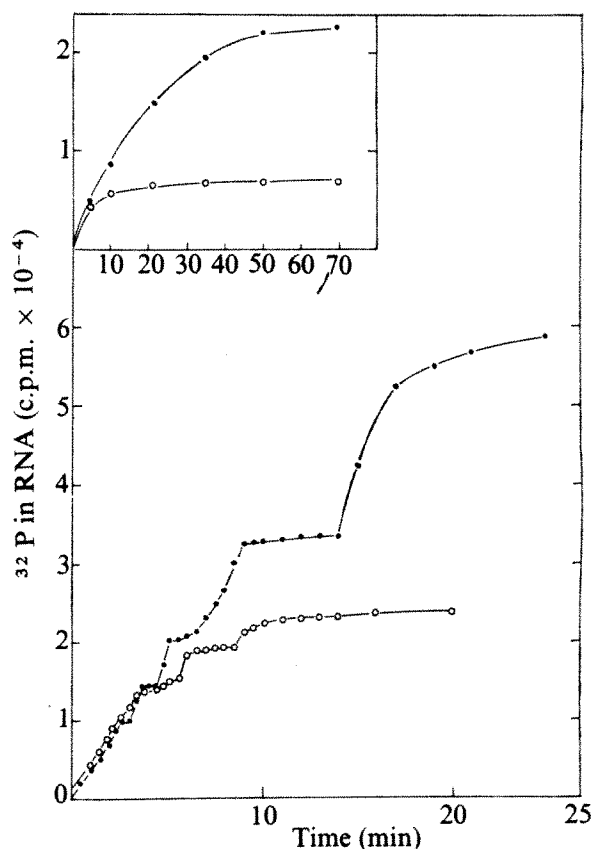
Fig. 1 Discontinuous transcription of T7 DNA *in vitro*. T7 DNA, *E. coli* RNA polymerase and factor p were prepared as described previously[7]. The reaction mixture contained 52 µg T7 ^{3}H–DNA (4,000 c.p.m. µg^{-1}), 10 µg RNA polymerase (50–70% active based on the specific activity reported by Burgess[11]), 7 µg p when added (○), 0.1 mM ATP, CTP, α-^{32}P-UTP (300 mCi mmol^{-1}), 0.015 mM GTP and salt conditions as described previously[7], final volume 2.5 ml. Where indicated, 75-µl aliquots were withdrawn and RNA recovered by acid precipitation. Insert, general aspect of the kinetics of RNA synthesis with T7 DNA template at limiting GTP concentration. In these conditions, the relative molar ratios of RNA polymerase to p and T7 DNA are 6:14:1 assuming respective molecular weights of 490,000, 250,000 and 25×10^6. ●, RNA made in absence of p; ○, in presence of p.
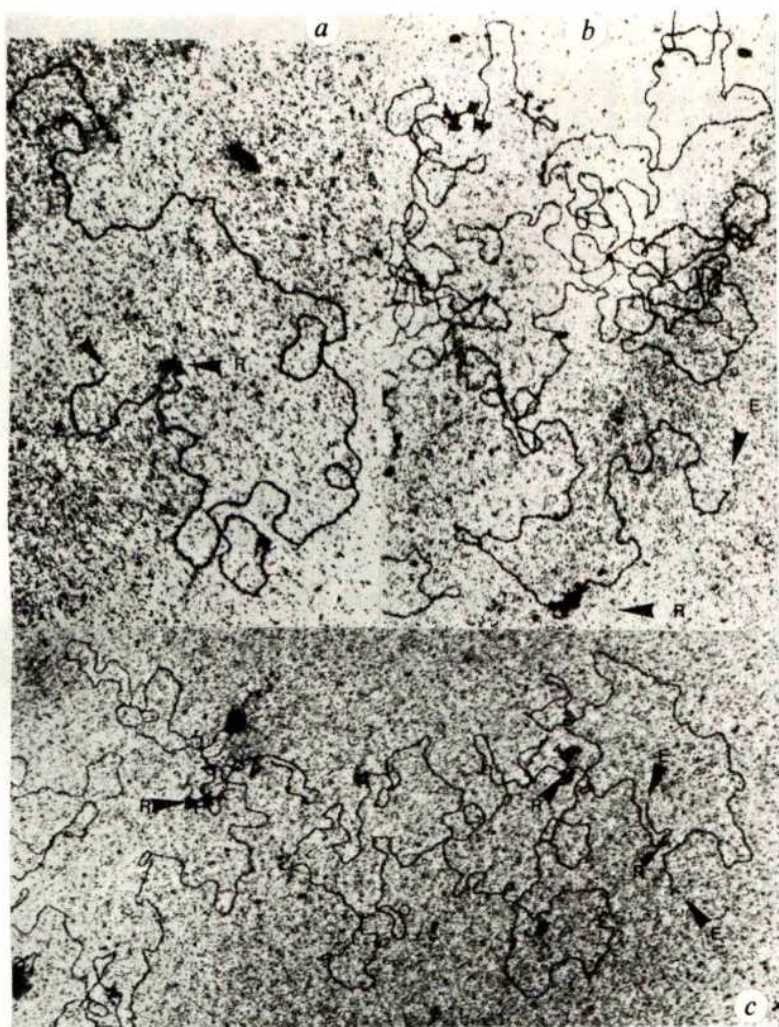
Fig. 2 Discontinuous transcription observed using electron microscopy. Conditions of transcription are those in Fig. 1. The samples were diluted to a concentration of 0.1 to 0.5 µg ml^{-1} T7 DNA with an ice-cold buffer (that of transcription) and specimens prepared as described previously[8]. Observations were made with a Siemens Elmiskop 101 electron microscope. The dark field was obtained by the tilted beam method and a thin-film objective aperture of 20 µm was used. Micrographs were recorded on 65×90 mm Kodak films at magnification ranging from 13,000–40,000. *a*, After 4 min transcription, small RNA bushes are visible. Magnification: ×80,000. E, DNA end; R, RNA bushes. *b*, After 11 min transcription, two DNA molecules are spread in the field and several RNA bushes are clustered at the end of gene 0.7. Magnification: ×65,550. *c*, After 4 min transcription in conditions which all four nucleotides were present at 0.1 mM. One entire DNA molecule is spread in the field and most RNA bushes visible are at the end genes. Small RNA bushes may be seen near one DNA end. Magnification: ×51,300.

were synthesised after, respectively, 4, 10 and 20 min of transcription at low GTP concentration. Consequently, sequences located between genes in the T7 DNA early region behave as transient transcriptional barriers. In addition, transcribing RNA polymerases also stop in the middle of gene 1, indicating the presence of a transcriptional barrier in this region[7]. In the present conditions total RNA synthesis ceases after 50 min and the major product is the polycistronic mRNA of T7 DNA early region as usual[7] (Fig. 1, insert).

On the basis of the above data we can estimate the relative times devoted to high and low rates of transcription and RNA chain growth rate. During 25 min of transcription, efficient RNA synthesis occurred during approximately 8 min (Fig. 1); thus a third of the time is devoted to efficient synthesis. These 8 min should be considered as a maximum, for the first 2 min of RNA synthesis are devoted to initiation (result not shown). As DNA sequences of 2,500 to 6,500 nucleotides have been transcribed within these 6–8 min of efficient synthesis (Fig. 3c), RNA chain growth rate varies from 5 to 18 nucleotides s^{-1}. Furthermore, in 3 min (third to fourth plateau, Fig. 1) RNA polymerase molecules can transcribe a sequence 3,600 nucleotides long, that of genes 1 and 1.1 (Fig. 3b and c), then at a chain growth rate of 20 nucleotides s^{-1}. Transcription of gene 0.7 proceeds at the same speed (second to third plateau, only a small fraction of enzymes have reached the end of gene 0.7 at 7 min; result not shown). Finally, assuming that all enzymes transcribe the genes at 20 nucleotides s^{-1}, the most efficient have read four genes (0.3 to 1.1; 5.5 min required) after 25 min synthesis, whereas

the less efficient enzymes have read only two genes (0.3 and 0.7; 2 min required).

On the other hand, transcription of the sequences located at the end and/or beginning of the early genes seems to proceed slowly and last a long time. If we estimate that the length of each transcriptional barrier does not exceed 190–285 nucleotides (0.50–0.75 T7 DNA map unit, Fig. 3 and ref. 10), the corresponding RNA chain growth rate should be about 10–60 nucleotides min^{-1}.

Thus all these calculations show that, at limiting GTP concentration, efficient transcription may well correspond to the rapid and brief synthesis of specific RNAs and low rate of transcription to the slow growth of short and particular RNA sequences[4,7,10]. A similar transcription pattern can be drawn from results of experiments in which the limiting nucleotide was CTP or UTP instead of GTP (results not shown). If the present conclusions are independent of the low concentration used for one nucleotide, a large fraction of the polymerases should also be found between genes after a given period of synthesis carried out at 50–100 nucleotides s^{-1}, that is, when the concentration of each nucleotide is 0.1 mM (ref. 6). In fact, after 4 min transcription with T7 DNA template, growing RNA transcripts are clustered near the end of genes, mostly at the end of gene 0.7 (Fig. 3d). Chain length analysis of the transcription product agree with the present findings, as most RNA are 2,300–3,000 nucleotides long corresponding to the transcription of genes 0.3 and 0.7 (results not shown). At a chain growth rate of 50–100 nucleotides s^{-1} (ref. 6), transcription of genes 0.3 and 0.7 should have required 30–60 s of the 4-min synthesis; thus the relative time de-
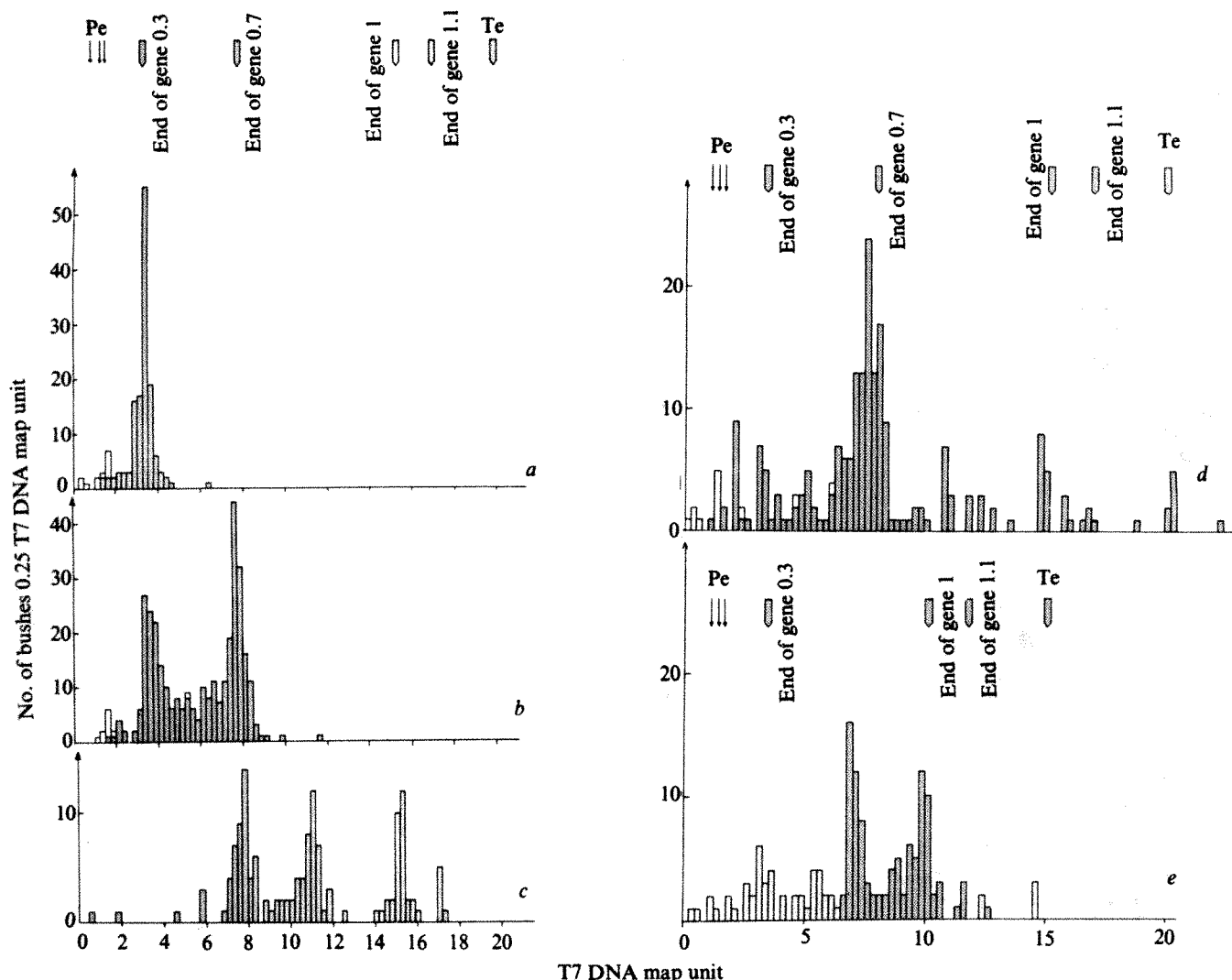
Fig. 3 Histograms of the positions of the transcription complexes. Experiments were carried out as described in Figs 1 and 2. Interesting features of the micrographs taken were redrawn on paper at ×10 magnification using an Automega enlarger. Length measurements were made on these drawings with a map ruler. One T7 DNA map unit corresponds to 380 base pairs. Mapping was according to Simon and Studier[9]. *a*, After 4 min transcription, 146 RNA bushes were on 62 DNA molecules. 58 free DNA ends observed. *b*, After 11 min transcription, 328 RNA bushes were on 135 DNA molecules. 100 free DNA ends. *c*, After 25 min transcription, 140 RNA bushes were on 53 DNA molecules. 50 free DNA ends. *d*, After 4 min transcription in conditions in which all four nucleotides were at 0.1 mM, 230 RNA bushes were on 79 DNA molecules. 70 free DNA ends. *e*, As in *d* but the template used was T7 C18 DNA, 153 RNA bushes were on 50 DNA molecules. 40 free DNA ends. Open areas, complexes in which RNA is hardly visible; stippled areas, complexes in which RNA is clearly visible.

voted to rapid transcription is roughly the same whether GTP concentration is limiting or not, and equal to 10–25% of the total transcription time.

With T7 C18 DNA template (in which regions of gene 0.7 and between 0.7 and 1 are deleted[8]) most transcribing RNA polymerases are not located in the same regions of the T7 DNA physical map, but again are still close to the end of genes and in the middle of gene 1 (Fig. 3*e*). After 15 min transcription and in conditions in which the RNA polymerase is in large excess, the growing RNA transcripts are mostly at the end of genes[12].

Transcriptional barriers associated with termination of RNA synthesis

We have shown above that regions located between genes behave as transient transcriptional barriers. In the absence of *ρ*, spontaneous termination of RNA synthesis seldom occurs at these sites[13] and they are transcribed. The double-stranded RNA that results is further digested by RNaseIII during the processing of the mRNA of the T7 DNA early region[4,10,14,15].

Addition of factor *ρ* to the transcription apparatus does not modify the binding of RNA polymerase to the DNA (ref. 2). As shown by electron microscopy, at respective molar ratios of RNA polymerase to *ρ* and T7 DNA of 6 : 14 : 1, three to four enzymes bind to the three to four sites located in the early promotor region at 1.1, 1.41, 1.65 and 0.5 units of T7 DNA physical map[2] (Figs 4 and 5). RNA synthesis very probably starts from these sites[7] and proceeds discontinuously in the presence of *ρ* (Fig. 1). In conditions of limiting GTP concentration, inhibition of transcription caused by *ρ* begins after 4–5 min; at that time most active enzymes have transcribed gene 0.3 and are between genes 0.3 and 0.7, where factor *ρ* specifically terminates transcription for the first time[7]. After 11 min of synthesis, termination of transcription intervenes once more between genes 0.3 and 0.7 and between genes 0.7 and 1. Transcription with *ρ* is completed after 20 min (Fig. 1, insert) and the major products are monocistronic mRNA of gene 0.3 (plus a part of the early promotor region) and of

gene 0.7 in a molar ratio 4 : 1 (Figs 4 and 5 of ref. 7).

The action of ρ may be seen clearly in Figs 4b and 6a, which show the localisation of RNAs still attached to the DNA after 11 min of transcription. The number of RNA bushes per T7 DNA molecule is two- to threefold less with termination factor ρ than without; RNA bushes are no longer clustered (Fig. 4b). An unusual number of RNA polymerases that have just initiated transcription are present in the regions of the early promotor and end of genes 0.3 and 0.7 (Fig. 6a). Remaining RNAs are not at the end of genes 0.3 and 0.7, but located between early promotor and the end of gene 0.7 and primarily within gene 0.3. Transcription complexes are also present between genes 0.3 and 0.7 indicating that ρ is not completely efficient and/ or part of the sequences at the end of genes are transcribed in the presence of ρ. Similar conclusions can be drawn from the distribution pattern of the RNA bushes after 4 min transcription with ρ in the presence of saturating concentration of the four nucleotides (Fig. 6c, for control see Fig. 3d).

Our findings establish certain aspects of the proposed mechanism for T7 DNA transcription with ρ. Transcription starts within the early promotor region, very probably at the three binding sites; data presented in Fig. 6 support the idea that reinitiation of transcription at the early promotor and at the beginning of genes 0.7 and 1 (ref. 16) follows the termination of transcription maximised by ρ at the end of genes 0.3 and 0.7 (refs 7, 13, 16). As there is no preferential degradation of one or more early T7 mRNA *in vivo*, our data clearly indicate a possible way in which transcription of the T7 early genes are modulated *in vivo*[17-19]. RNA transcripts made in the presence of ρ

Fig. 5 Position of the RNA polymerase binding sites in T7 DNA early promotor region in presence of ρ. Specimens were prepared and analysed as described in Figs 3 and 4. Histogram of the positions of 114 RNA polymerase molecules on 38 T7 DNA molecules. One T7 DNA unit corresponds to 380 base pairs.

are processed by RNaseIII to give monocistronic mRNA of T7 DNA early region identical to those found *in vivo* and in similar relative ratios[7].

Finally, the action of ρ between genes is well shown in Fig. 6b. After transcription without ρ and at limiting GTP concentration, ρ is added (plateau, Fig. 1). One minute later the synthesis is stopped and the distribution pattern of the growing RNA chains determined. Almost all RNA transcripts clustered at the end of genes 0.3 and 0.7 have disappeared (70% total RNA chains), whereas those within gene 0.3 remain (for control, see Fig. 3b).

Conclusions

It now seems possible to draw the following picture for the *in vitro* synthesis of a polycistronic mRNA such as that of the T7 DNA early region. Once RNA chain initiation is completed, intense bursts of transcription of long DNA sequences, the genes, alternate with the slow synthesis of short and particular RNA sequences which takes a long time to complete.

On the basis of our experiments and on those of others (for review see ref. 1), we may well understand the mechanism of *in vitro* ρ-dependent termination of transcription. ρ Termination is directly related to the decrease in RNA chain growth rate at the end of genes and in the middle of gene 1. Furthermore, efficiency of ρ terminations is also related to the time necessary for RNA polymerase to read through a transcriptional barrier. When the mean chain growth rate is decreased by limiting concentration of one nucleotide, transcription beyond gene 0.7 does not occur, or at least is extremely low[7]. In addition, all attempts made to demonstrate the presence of ρ-specific binding sites on T7 DNA, as on fd DNA[20], were unsuccessful. Intervention of ρ during transcription may well seem to be a probable and transient interaction between this factor and slowly transcribing RNA polymerases, the conformation of which has been modified by a transcriptional barrier.

Such T7 DNA sequences, which include ρ-active sites, also coincide with the corresponding RNaseIII cleavage sites, of the RNA transcript[4,7,14]. Data now available show that of these DNA sequences some should be $(dA)_n$: $(dT)_n$-rich[10,21] and other $(dC)_n$: $(dG)_n$-rich[10], and expected to be

Fig. 4 Transcription of T7 DNA in presence of factor ρ observed using electron microscopy. a, Binding complexes. Conditions of binding were as reported in Fig. 1 except that nucleotides were absent. After 5 min incubation at 37 °C dilution of the samples and specimen preparation were carried out as described in Fig. 1 but at 37 °C instead of 0–4 °C. Two individual DNA ends are visible with RNA polymerase molecules either bound near DNA end or in the background of the film. Magnification: ×79,160. b, Transcription complex. Conditions of transcription with ρ were as reported in Fig. 1. A typical RNA bush bound to DNA is shown. Magnification: ×93,800. E, DNA end; R, RNA bush; P, RNA polymerase.

Fig. 6 Positions of the transcription complexes in presence of ρ. Experiments were carried out as in Figs 1–3. *a*, ρ added together with RNA polymerase, and transcription was stopped after 11 min. 120 RNA bushes were on 96 DNA molecules, 100 free DNA ends observed. *b*, ρ was added after 10 min transcription, itself stopped 1 min later. 121 RNA bushes were on 64 DNA molecules. 110 free DNA ends. *c*, ρ was added together with RNA polymerase, and transcription was carried out in conditions in which all four nucleotides were at 0.1 mM. 140 RNA bushes were on 100 DNA molecules. 100 free DNA ends. Open areas, complexes in which RNA is hardly visible; stippled areas, complexes in which RNA is clearly visible.

preceded by a specific sequence –G–C–C–U–U–U–A–U– – and followed by –G–A–U– (ref. 22).

More generally, similar transcriptional barriers probably exist in all natural DNA as indicated by the heterogeneous distribution of the RNA made in the course of transcription with T4, φ80, λ, *E. coli* and calf thymus DNA templates[7], and these barriers exert a control on transcription in the presence or absence of an additional factor[1] We have shown termination of T7 DNA transcription under the control of ρ; this process has also been described with various natural DNAs but not with artificial templates[1] and thus seems to be a general phenomenon requiring specific DNA sites and ρ. Another termination event occurs at the end of the early region of T7 DNA and is ρ independent[23]; the barrier located at 20% of the T7 DNA physical map, however, does not stop all transcriptions and its efficiency decreases with increasing concentration of salt[16]. Such transcriptional barriers are not rare and exist within the late region of T7 DNA[23], and also within λ and *E. coli* genomes[24,25].

Finally, transcriptional barriers can be located very close to the promotor region of a bacterial operon, and could thus be important in the control of transcription of one or more structural genes. In the course of transcription of the tryptophan operon, RNA polymerase is frequently reiected at a specific site ahead of the first structural gene, but a protein factor was found to antagonise the abortive synthesis of tryptophan mRNA[26].

We thank C. Bardin for assistance; I. Pinset, J. Dubochet and M. Schweiger for suggestions; B. Haas. A. Sentenac for reading the manuscript; and P. Fromageot for his interest.

1. Chamberlin, M. J., *A. Rev. Biochem.*, **43**, 721–775 (1974).
2. Bordier, C., and Dubochet, J., *Eur. J. Biochem.*, **44**, 617–624 (1974).
3. Chamberlin, M. J., and Ring, J., *J. molec. Biol.*, **70**, 221–237 (1972).
4. Dunn, J. J., and Studier, F. W., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1559–1563 (1973).
5. Darlix, J. L., and Dausse, J. P., *FEBS Lett.*, **50**, 214–218 (1975).
6. Darlix, J. L., Sentenac, A., and Fromageot. P., *Biochim. biophys. Acta*, **166**, 438–449 (1968).
7. Darlix, J. L., *Biochimie*, **56**, 693–703 (1974).
8. Dubochet, J., Ducommun, M., Zollinger, M., and Kellenberger, P., *J. Ultrastruct. Res.*, **35**, 147–167 (1971).
9. Simon, M. N., and Studier, F. W., *J. molec. Biol.*, **79**, 249–265 (1973).
10. Darlix, J. L., and Fromageot, P., *Biochimie*, **56**, 703–710 (1974).
11. Burgess, R. R., *J. biol. Chem.*, **244**, 6160–6169 (1969).
12. Delius, H. Westphal, H., and Axelrod, N., *J. molec. Biol.*, **74**, 677–687 (1973).
13. Minklev. E., *J. molec. Biol.*, **83**, 305 (1974).
14. Dunn, J. J., and Studier, F. W., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 3296–3300 (1973).
15. Darlix, J. L., *Eur. J. Biochem.*, **51**, 369–376 (1975).
16. Darlix, J. L., *Eur. J. Biochem.*, **35**, 517–526 (1973).
17. Summers, W. C., Brunovskis, I., and Hyman, R. W., *J. molec. Biol.*, **74**, 291–300 (1973).
18. Ponta, H., *et al.*, *Molec. gen. Genet.*, **134**, 281–297 (1974).
19. Minkley, E., *J. molec. Biol.*, **83**, 289–304 (1974).
20. Oda, T., and Takanami. M., *J. molec. Biol.*, **71**, 799–802 (1972).
21. Gomez, B., and Lang, D., *J. molec. Biol.*, **70**, 239–251 (1972).
22. Rosenberg, M., Kramer, A. R., and Steitz, J. A., *J. molec. Biol.*, **89**, 777–782 (1974).
23. Golomb, M., and Chamberlin, M., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 760–764 (1974).
24. Lebowitz, P., Weissman, S. M., and Radding, C. M., *J. biol. Chem.*, **246**, 5120–5139 (1971).
25. Ikemura, T., and Dahlberg, J. E., *J. biol. Chem.*, **248**, 5033–5041 (1973).
26. Pannkoek, H., Brammar, W. J., and Pouwels, P. M., *Molec. gen. Genet.* (in the press).

letters to nature

Hard X-ray observations near Ariel 1118 – 61

OBSERVATIONS made by Ariel V in the region of Cen X-3 have revealed a new transient X-ray source with a periodicity of 6.75 min (refs 1 and 2). Tentative identifications are: with a Mira-type variable star by Fabian *et al.*[3], who suggest the 6.75 min periodicity is associated with the rotation period of a compact companion; and with a heavily reddened, variable

ultraviolet star[4]. As well as the proportional counter observations during the period 1974 December 17 to 1975 January 31, the Imperial College crystal scintillator, sensitive to the photon energy range 26 keV to 1.2 MeV, viewed the same region of the sky. This detector has a sensitive area of 8 cm², an opening angle of 8° FWHM defined by an active collimator and is offset from the spacecraft spin axis by an angle of 3°. A source a few degrees from the direction of the spin axis can be detected

by determining the modulation in the counting rate seen in four equal angle spin sectors with a time resolution of 512 s. A single channel (50–190 keV) mode with a 48 s time resolution was also used. Since a spurious modulation is induced in the apparatus by cosmic rays, two pointing direction offsets on opposing sides of the source have to be used, and the X-ray contribution is obtained by a subtraction technique.

Scheduled observations of Cen X-3 coincided with an extended low from 1974 December 17 to 1975 January 10 and no detectable signal was seen above statistical noise. The offsets were, however, equally good for putting upper limits on the hard flux of A1118−61 (Table 1).

Table 1 Upper limits on A1118−61 flux

Period	2σ upper limits (photons cm^{-2} s^{-1} keV^{-1}) 40 keV	80 keV	180 keV
1974 December 19–22	3.4×10^{-4}	1.8×10^{-4}	4.0×10^{-5}
1975 January 8–11	5.4×10^{-4}	1.7×10^{-4}	7.8×10^{-5}

To compare these results with those reported at lower energies, we note that Ives *et al.*[2] find the spectral shape varies little during their observations using proportional counters. We can therefore scale the proportional counter spectrum given by these workers, according to the light curves of references 1 and 2 to obtain the low energy spectrum corresponding to our periods of observations. Figure 1 shows the result of this procedure with the proportional counter points reduced by a factor of 2.3 to correspond to the epoch 1974 December 19–22. Our 2σ scintillator upper limits are plotted at the logarithmic

Fig. 1 Four independent, hard X-ray upper limits plotted at 2σ for the spectrum of A1118−61 together with low energy X-ray data normalised to the same epoch (1974 December 19–22). Three theoretical fits to the data are also shown: *a*, 3 keV black body spectrum; *b*, 7 keV black body spectrum modified by electron scattering; *c*, 20 keV thermal bremsstrahlung spectrum +, proportional counter data; ○, offset mode; ☐, search for 6.75 min period.
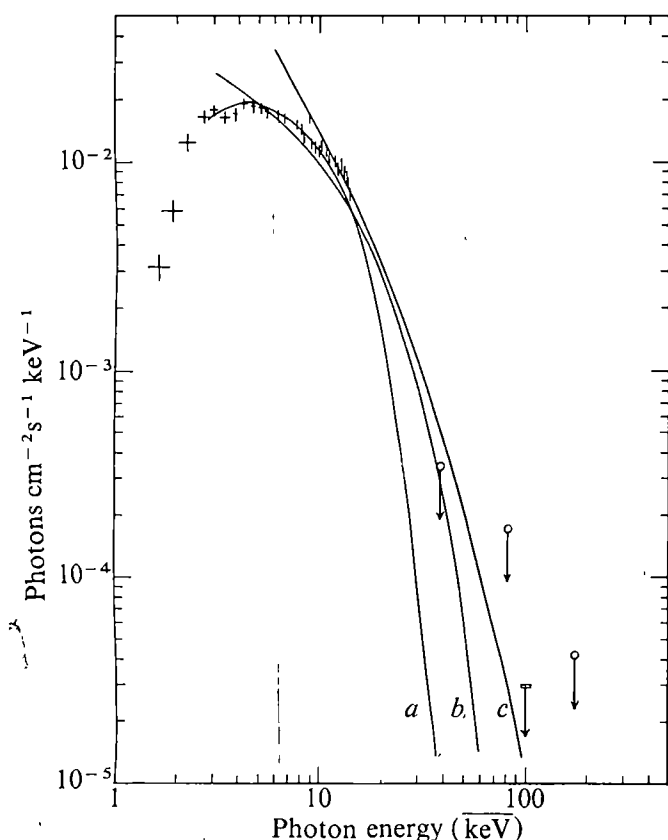
mean energy of the channels and are virtually independent of the assumed spectral slope within the channel.

A further independent upper limit for the 6.7506 ± 0.006 min periodicity (J. Bell Burnell, personal communication) has been obtained using observations with the 48 s time resolution mode made in the period 1975 January 19–27. These data, which are less sensitive to the cosmic ray induced background, were folded with the quoted period. A 2σ upper limit for the depth of modulation of 3×10^{-5} photons cm^{-2} s^{-1} keV^{-1} was obtained. This value, which has been normalised to a photon energy of 100 keV, is almost independent of the assumed spectral shape within the energy window for a range of likely cosmic spectra. Assuming there is essentially 100% modulation as indicated in reference 2, the above limit must be approximately halved for comparison with the time averaged data. But since the intensity may be a factor of two lower for this observation than for the standard epoch (that is, 1974 December 19–22) we plot 3×10^{-5} photons cm^{-2} s^{-1} keV^{-1} as the corrected 2σ upper limit in Fig. 1.

In discussing the physical processes for the emission implied by the combined spectral measurements, it is clear that any power law spectrum fitted above 10 keV must be steeper than E^{-3}, although the published low energy data enabled even an E^{-1} fit. Thus there is no immediate reason to consider a synchrotron source. Instead, consider accretion on to a compact stellar object in a binary system. Indeed we note that the rapid spectral cutoff below 4 keV suggests strong local gas absorption[2]. The dominant mechanism for emission of X rays from the gas clouds accreting on to the compact object could be either (a) a black body radiation where photon scattering by electrons is unimportant, or (b) a modified black body radiation at a higher temperature with a significant skin depth so that photons must random walk. We may further modify (b) by adding a high temperature bremsstrahlung tail (free-free emission) due to inner regions of the accretion cloud shining through at harder X-ray energies. Our best fit to hypothesis (a) using a photon spectrum

$$(dI/dE)_{BB} \propto E^2/(\exp(E/kt)-1)$$

yields $kT = 3 \pm 1$ keV and this is plotted in Fig. 1 as curve *a*. Fitting a modified black body spectrum (curve *b*) to the proportional counter data using

$$(dI/dE)_M \propto (dI/dE)_{BB}[gE^{-3}(1-\exp(-E/kT))]^{1/2}$$

with the Gaunt factor $g \propto E^{-0.4}$ gives $kT = 7$ keV ± 1 keV. But plotting this spectrum in Fig. 1 results in a curve which is a poor fit to the lowest in energy of the scintillator counter 2σ upper limits and does not satisfy the proportional counter data so well. A thermal bremsstrahlung spectrum (*c*) with $(dI/dE)_{TB} \propto E^{-1.4} \exp(-E/kT)$ and $kT \sim 20$ keV is a plausible fit to the higher energy proportional counter data but is similarly inconsistent with the hard X-ray data. We conclude that the addition of scintillator X-ray data to the A1118−61 observations suggests that if a dominant, single source mechanism is sought, black body radiation from a low skin depth gas is the most likely.

G. F. CARPENTER
M. J. COE
A. R. ENGEL
J. J. QUENBY

Physics Department,
Imperial College,
London SW7 2AZ, UK

1 Eyles, C. J., Skinner, G. K., and Willmore, A. P., *Nature*, **254**, 577 (1975).
2 Ives, J. C, Sanford, P. W., and Bell-Burnell, S. J., *Nature*, **254**, 578 (1975).
3 Fabian, A C., Pringle, J. E, and Webbink, R. F, *Nature*, **255**, 208 (1975).
4 Chevalier, C., and Ilovaisky, S. A., *IAU Circ. No.* 2778, May 9 (1975).
5 Felten, J. E., and Rees, M. J. *Astr. Astrophys.*, **17**, 226 (1972).

Radio emission from Cyg X-3

THE most recent radio outburst of Cyg X-3 reported[1,2] occurred in May, 1974. Following the announcement by Ryle[1] of high activity we observed part of this outburst at 4.8 GHz with the 100-m telescope of the MPIfR at Effelsberg from May 22 to May 31, during a general survey of the Cygnus X region. The observed flux densities are summarised in Fig. 1 and have typical errors of 3% or less; the general behaviour of the sources is also sketched in
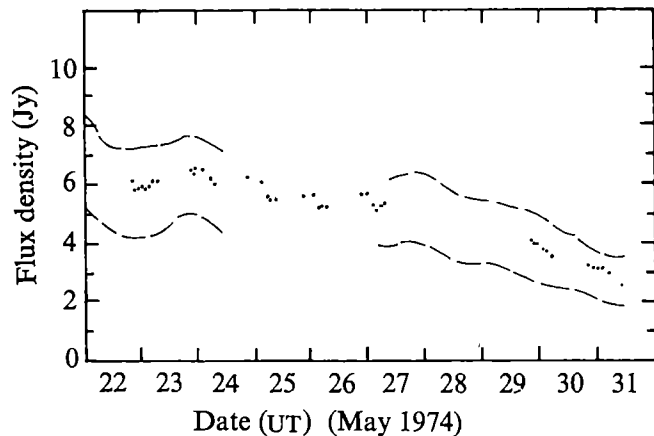
Fig. 1 Light curve of Cyg X-3 at 4.8 GHz (filled circles). The dashed lines sketch the data from Seaquist *et al.*[2].

for the data given by Seaquist *et al.*[2]. The observations were obtained as a flux density ratio with respect to NGC7027 from direct adjacent crosscuts, assuming a value of 5.8 Jy for NGC7027 (from refs 3 and 4). As our data did not contain very extended polarisation measurements and as they are consistent with the interpolated polarisation properties given in ref. 2, all points were brought to the mean flux density level using a polarisation angle of 70° and a linear polarisation of 4.7%.

Three important results emerge from our study. First, the source also shows the linear decay of long time averaging over the missing time interval of ref. 2. (For a discussion of related problems see ref. 5.) Second, the periodicity indicated in the detailed flux density behaviour is also visible in our data. Moreover we have the impression that this periodicity becomes less pronounced when progressing from May 22 to May 31. Third, we emphasise that it may be dangerous to infer a 'linear' spectrum from only two points. In Fig. 2 several spectra are shown constructed from the data of

ref. 2 and our observations. There is a strong indication that the assumption of optically thin properties may not be valid even during the decay of this outburst. This does not seem to be a systematic calibration effect between the two sets of observations.

Forcing our data down to an interpolated value on the 'linear' spectra would require us to assume for NGC7027 at 4.8 GHz a flux density value of 5.3 Jy or less which seems completely out of the question according to the spectrum given by Higgs[3] and Ross and Seaquist[4]. From a few flux density ratio measurements NGC7027/3C48 we derived a value of 5.4 ± 0.3 Jy for 3C48 which is compatible with an interpolated value used in ref. 2. Thus, during the time May 22 to May 31 the spectra may be interpreted as showing the effects of optical depth (see ref. 6). A possible explanation of this behaviour may be that the sum of the recent outbursts has accumulated enough thermal plasma on the outskirts of the source for absorption now to occur even at frequencies up to several GHz.

The activity of Cyg X-3 has been quite high during the past few years and it is almost possible to say that the source underwent an outburst once every month. The source was visible for about a couple of weeks at an appreciable flux density level during each flare. So the question of past activities of the source may be important for all explanations of the nature of the source. One of us (H.J.W.) has obtained many observations of the area during regular surveys of the

Table 1 Older data for Cyg X-3

Date	Frequency (GHz)	Upper limit (Jy)	Telescope
1964			
July 4	2,695	2.0	NRAO 26 m
July 5	2,695	2.0	
July 26	2,695	2.0	
July 31	2,695	2.0	
1965			
January 13	4,930	4.0	NRAO 26 m
January 14	4,930	4.0	
January 20	4,930	4.0	
1967			
May 31	2,695	0.3	NRAO 45 m
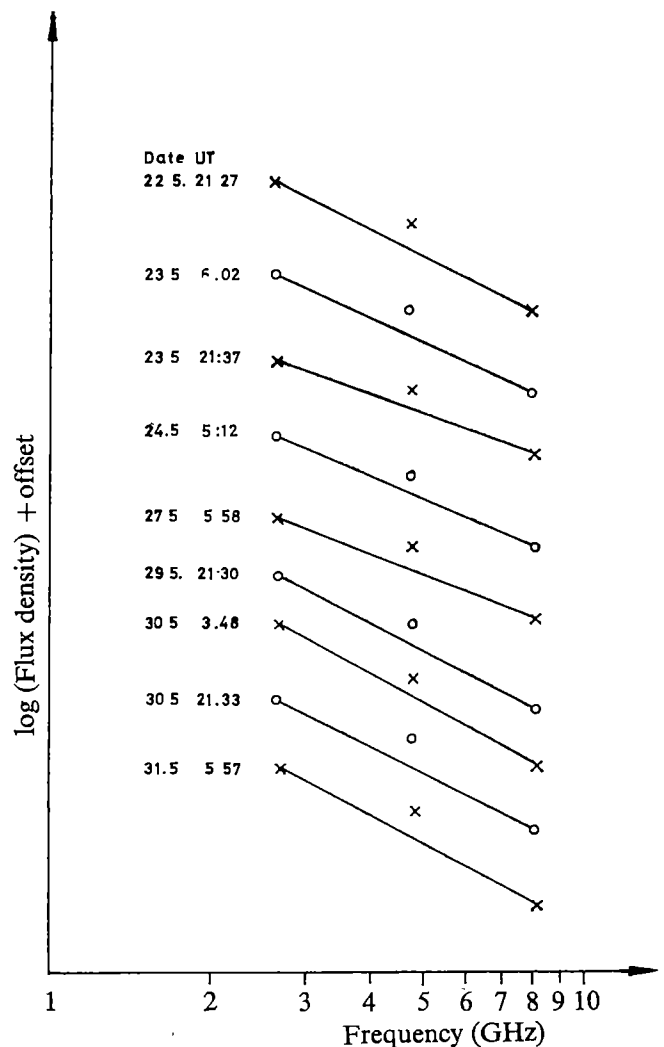
Fig. 2 Spectra of Cyg X-3 on the dates indicated.

Cygnus X region[7,8]; activity at the present level had a high chance of being discovered. But the data would have been regarded as showing a spurious source. So we re-examined the old records. The dates of observations and the derived upper limits (3σ) are given in Table 1. Especially during July 1964 and January 1965, outbursts such as, for example, those occurring in September 1972 and in May 1974 would easily have been seen. We are tempted to conclude that ten years ago the source was not so active as today.

H.-D. BOHNENSTENGEL
K. HENNING
H. J. WENDKER

Hamburger Sternwarte,
Gojenbergsweg 112,
2050 Hamburg 80, West Germany

1 Ryle, M., *IAU Circ. No. 2670* (1974).
2 Seaquist, E. R., *et al.*, *Nature*, 251, 394-396 (1974).
3 Higgs, L. A., *Publ Astrophys. Branch*, 1, 1-454 (1971).
4 Ross, H. N., and Seaquist, E. R., *Mon. Not. R. astr. Soc.*, 170, 115-119 (1975).
5 Marsh, K. A., Purton, C. R., and Feldman, P. A., *Astrophys. J.*, 192, 697-701 (1974).
6 Gregory, P. C., and Seaquist, E. R., *Astrophys. J.*, 194, 715-723 (1974).
7 Wendker, H. J., *Veröff. astr. Inst. Univ. Munster*, No. 10 (1966).
8 Wendker, H. J., *Astr. Astrophys.*, 4, 378-386 (1970).

The 'spectra' of the solar cycle and of data for Atlantic tropical cyclones

TROPICAL cyclones (including hurricanes) are significant climatic features which affect the South and East USA as well as areas of the western Atlantic, the Caribbean, and the Gulf of Mexico. Since they are considered to be a mechanism which limits the build-up of heat and energy in tropical regions[1], cyclones are necessarily related to large scale circulation patterns which may be global in extent. Thus, if a relationship is to be found between solar activity and large scale meteorological phenomena, we may expect that it will evidence itself in analyses of data on cyclone occurrence and the length of the cyclone season. Here we report results of such a study, and provide evidence which is consistent with the hypothesis that a relationship exists between the solar cycle and the occurrence of Atlantic tropical cyclones.

The data sets analysed[2,3] relate to tropical cyclones which originated in the Atlantic region during each month from 1871 to 1973 (see ref. 2 for a discussion of the sources and quality of the cyclone data). Both the number of cyclones which occurred in each year, and the length of the annual cyclone season in months (counted as the number of months from the month of the first cyclone to the month of the last cyclone, inclusive), were extracted from the data and were smoothed with a 7-yr running mean filter (Fig. 1a, b). Also shown in Fig. 1 are the 12-month running mean sunspot numbers[4,5] (Fig. 1c). It was necessary to smooth the cyclone data to ensure that scatter in the data did not cause the maximum entropy spectral analysis (MESA) technique (based on an autoregressive description of the data by Burg (unpublished); see also ref. 6) to fail to give acceptable spectral estimates[7].

MESA spectra for the smoothed number of Atlantic tropical cyclones and the smoothed length of the cyclone season are shown in Fig. 2a and b. For comparison, the MESA spectrum for the 12-month running mean sunspot numbers[8] (1750-1963) is given in Fig. 2c. Use of a longer time period in the analysis of the sunspot data is based on the assumption that all of the time series analysed here are stationary. Though spectral components determined using the MESA technique may be shifted somewhat in frequency[7], similarities in the spectra are evident. Note that long-period components in the spectra for the cyclone data
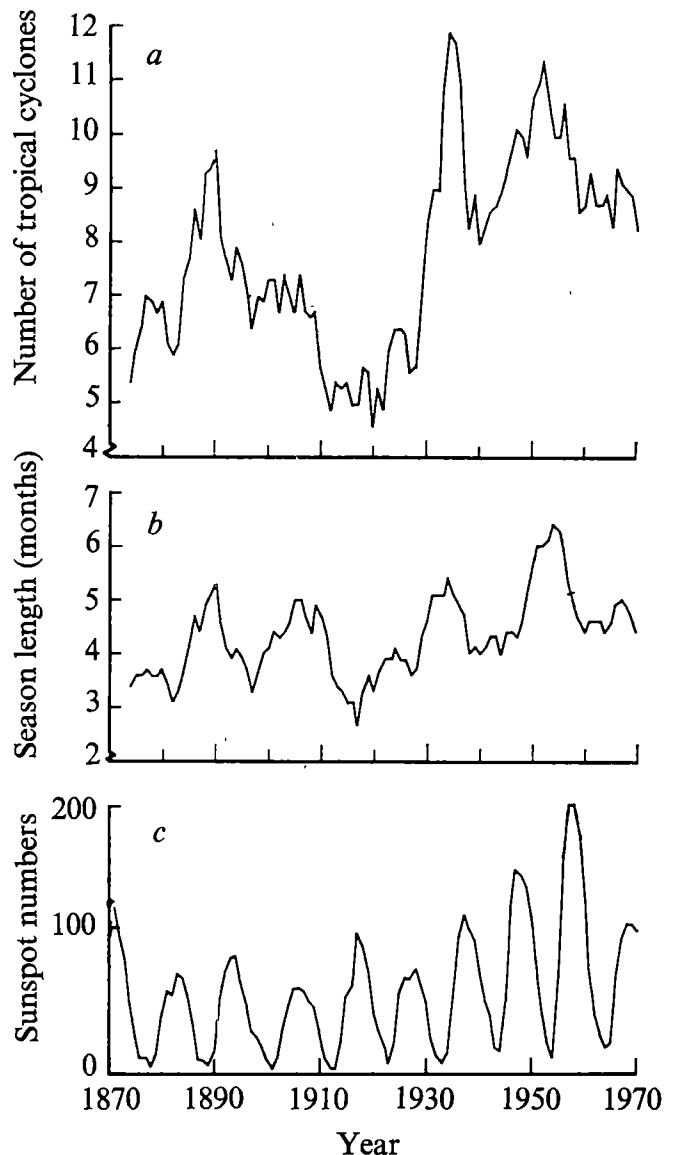
Fig. 1 *a*, 7-yr running mean number of Atlantic tropical cyclones; *b*, 7-yr running mean length of Atlantic tropical cyclone season; *c*, 12-month running mean sunspot numbers.

at 133 yr (Fig. 2a) and 154 yr (Fig. 2b) may represent ill-defined estimates for the 96 yr component found in the spectrum for the sunspot data. That these estimates are shifted to longer periods may result from the initial phase of the long period component in the window analysed or from the limited length of data available for analysis[7]. It is also possible that the data from 1871 to 1905 underestimate the number of cyclones which occurred each year as well as the annual lengths of the cyclone seasons, thereby introducing a long period bias into the data (after 1905, the use of radio aboard ships at sea greatly increased the quantity and quality of weather data acquired).

With respect to the 11-yr periodicities in tropical cyclone occurrence and length of season, it is interesting that some data for other meteorological phenomena exhibit this same periodicity. For example, Currie[9] observed a 10.6-yr periodicity in the spectra of surface air temperature data from North America, and concluded that solar activity has measurably affected this climatic element.

Although prominent spectral components at periods of 15 and 22 yr are found in the MESA spectra for the data on the length of the cyclone season, and appear only weakly in the sunspot spectrum, similarities in the MESA spectra
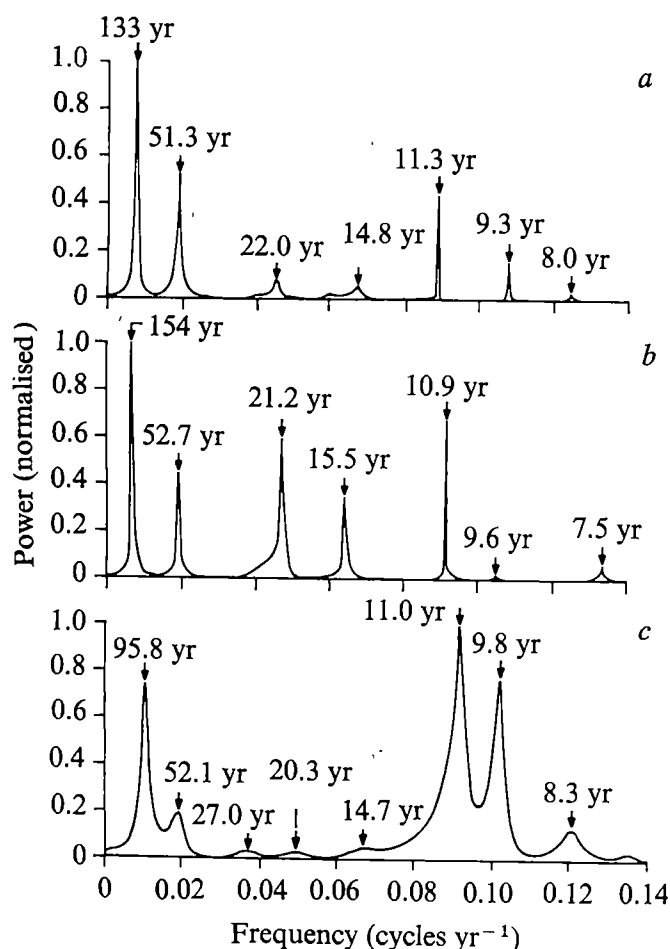
Fig. 2 Maximum entropy spectra for data shown in Fig. 1. *a*, Number of tropical cyclones; *b*, length of tropical cyclone season; *c*, sunspot numbers (spectra computed from data in the interval 1750–1963).

for the smoothed number of North Atlantic cyclones, the smoothed length of the cyclone season, and the 12-month running mean sunspot numbers suggest that a relationship exists between the solar cycle and Atlantic tropical cyclones. Whether the 22-yr periodicity found in the tropical cyclone data has its origin in the reversal of the magnetic fields associated with sunspots (the so-called "Hale double sunspot cycle") is speculative. The possibility that a long period resonance exists in the Earth's response to solar radiation must also be considered.

We offer no explanation as to how variations in the solar cycle influence the tropical cyclone phenomenon, and more generally, the climate.

We thank Dr R. Blandford and the referees for comments.

·Theodore J. Cohen
Erton I. Sweetser

*Teledyne Geotech,
Alexandria, Virginia 22313*

1 Landsberg, H E., *J. geophys. Res.*, 65, 1305–1307 (1960).
2 Cry, G. W., *Tropical Cyclones of the North Atlantic Ocean*, Tech. Paper 55 (Weather Bureau, US Department of Commerce, 1965).
3 Herbert, P J., *North Atlantic Tropical Cyclones, 1973*, from *NOAA Climatological Data, National Summary*, 24 (NOAA, Boulder 1973).
4 Waldmeier, M., *The Sunspot Activity in the Years 1610–1960* (Technische Hochschule, Zurich, 1961).
5 Waldmeier, M., *Monthly Sunspot Bulletin from the Swiss Federal Observatory* (Zurich, 1960–1972).
6 Ulrych, T. J., *J. geophys. Res.*, 77, 1396–1400 (1972).
7 Chen, W. Y., and Stegen, G. R., *J. geophys Res*, 79, 3019–3022 (1974).
8 Cohen, T. J , and Lintz, P. R., *Nature*, 250, 398–399 (1974)
9 Currie, R. G., *J. geophys. Res.*, 79, 5657–5660 (1974).

Odd and even numbered year summer temperature pulse in central England

Meteorologists and amateur weather observers with long memories have noted for some time that there seemed to be a preference for the hottest summers to occur during odd numbered years and the coolest during even numbered years during this century in south-eastern England. Many studies made during the past decade have supported the validity of such recollections for a much wider area. For example, Sutton[1] and Murray[2] have identified the existence of a biennial oscillation of the atmosphere and discussed its effects on the tropospheric circulation in both tropical and temperate latitudes. Davis[3] has found a high statistical significance in temperature differences between odd and even numbered years for an extensive area of western Europe.

The publication[4] of temperature observations for central England brings this long series up to date. The series, starting with 1721, has now been examined again to test whether the even–odd year cycle is peculiar to the summers of the present century, or whether it is indeed a long term feature of our climate. Data from years before 1721 have been excluded as they are less reliable. We have used the month of July; as the July temperature in England is highly correlated with sunshine and low rainfall it can be used as a good index of summer weather.

To remove the influence of longer period oscillations in the data, the anomalies in temperature for each year were obtained by using averages taken over decades. We have defined a tercile distribution of anomalies greater than + 0.4 °C, within ± 0.4 °C inclusive and less than − 0.4 °C. The distribution within these classes for even and odd years is shown in Table 1.

Table 1 Distribution of temperature anomalies in July (1721–1970)

	$A > + 0.4\,°C$	$B \pm 0.4\,°C$	$C < - 0.4\,°C$	Total
Even years	30	47	48	125
Odd years	51	26	48	125

The χ^2 test shows that the distributions are significantly different to the 1% level. The differences show that in 21 even Julys there is a shift from above average to average compared with the odd Julys: the probability of an above average July in an odd year is 0.41 compared with 0.24 in an even year. The probability of a below average July is 0.38 for both odd and even years.

The period from 1911 to 1970 was examined separately. The distributions are shown in Table 2. The probability of having an

Table 2 Distribution of temperature anomalies in July (1911–1970)

	$A > + 0.4\,°C$	$B \pm 0.4\,°C$	$C < - 0.4\,°C$
Even years	5	11	14
Odd years	14	9	7

above average July in an odd year has increased to 0.47 as against 0.17 for an even year. Likewise, the probability of a cool July in an even year has increased to 0.47.

The results show that in the whole period from 1721 to 1970 a highly significant biennial pulse is occurring in the July temperatures. The main characteristic of the pulse during the

past 60 yr is the occurrence of warm Julys in odd years and cool Julys in even years, though taken over the whole 250-yr period this characteristic is not so well marked.

A. H. GORDON
N. C. WELLS

Department of Geophysics,
University of Reading,
Whiteknights Park,
Reading, UK

[1] Sutton, O G., *Weather*, 17, 408 (1962)
[2] Murray, R., *Weather*, 27, 161–169 (1972).
[3] Davis, N. E., *Met. Mag*, *Lond.*, 96, 178–187 (1967).
[4] Manley, G., *Q. Jl R. met. Soc*, 100, 389–405 (1974)

Brine at the bottom of the Great Bitter Lake as a result of closing the Suez Canal

DURING the process of clearing the Suez Canal and the Bitter Lakes from bombs and mines resulting from the 1967 and 1973 wars, an abnormal phenomenon was observed. Divers reported that diving was rather difficult through a layer extending about two metres above the bottom in the eastern part of the Great Bitter Lake (GBL). When this layer was reached through diving, any diver was forced to turn upwards. Moreover, sounding of depths by the use of sonic waves gave values which were less by about 2 m than when measured by weight and line.

A preliminary investigation of salinity was made on June 20, 1974, in the GBL. This showed that the salinity near the bottom was exceptionally great in many locations, especially in the eastern area of the lake. One of these locations was selected for detailed study. It was 16.5 m deep and located at a distance about 4 km east of the navigation channel. Water samples were collected at surface, 6, 12, 14 and 16 m depths, and temperatures at every depth were measured. Hydrogen sulphide was smelt distinctly in the samples collected at and below 14 m depth where several rotten fish were found. Bottom samples were sludgy and had a black colour.

Salinity was estimated by two different and independent methods after diluting the water samples with distilled water: determination of chlorinity by the Knudsen titration method[1], in which chlorinity was converted to salinity using an empirical equation suggested by Morcos and Riley[2] for the Suez Canal region, and measurement of conductivity ratio. The two methods gave very close results.

Salinity and temperature values of the water column are given in Table 1; they show that, with increasing depth, the temperature variation is within the normal range while the increase of salinity is very rapid. A layer of brine (salinity more than 300‰) about 2 m thick exists above the bottom. This exceptionally high salinity had never been reported before.

The GBL was a dry basin before it was filled by water from the Mediterranean and Red Seas in November 1869. Its bottom is formed of multilayers of salt deposits separated by films of sand and gypsum. Early investigations[3] of the salinity of the Suez Canal and its lakes showed that even during the operation of filling the GBL with sea water, the highest salinity recorded was 168.6‰ (by evaporation) when water from the Mediterranean was covering the salt bed by only 1.4 m. Evidence based on observation[4-6] of salinity in 1924–25 and 1933–34, suggests that the southern part of the canal possessed higher salinity than the GBL itself. Observations during 1954 and 1955 indicate[7] that the maximum salinity in the Suez Canal
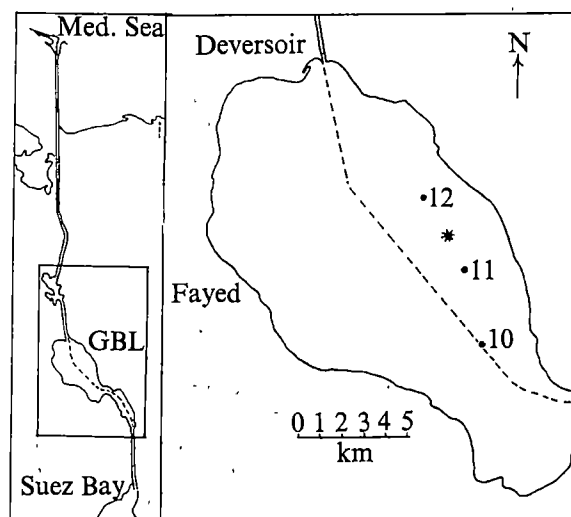
Fig. 1 Map of the Great Bitter Lake showing the locations of stations 10, 11 and 12 occupied by El-Sharkawy[8] in April 1967 and the position (*) where the samples were collected in June 1974.

region (48.4‰) was reached in late summer in the southern canal but not over the salt bed as might be expected. El-Sharkawy[8] carried out observations of salinity in the Bitter Lakes for one year (April 1966–April 1967) and the salinity in GBL did not exceed 49‰. His last cruise (April 1967) is of great interest since it was made only two months before the closing of the Suez Canal that June. The salinity–depth profile of the three stations 10, 11, 12, which were occupied in the eastern part of the GBL (Fig. 1) is reproduced in Table 2. His deepest samples were obtained at only 12 m depth, but the salinity was increasing gently with depth and did not show any indication of an abrupt increase. By contrast, my results (Table 1) show unusually high salinity at 6 m and approach saturation at 14 m. Comparison between Tables 1 and 2 shows that my surface salinity is even higher than that obtained at 12 m on the 1967 cruise. This clearly indicates that a rather abnormal phenomenon occurred in the GBL during the period of closure of the Suez Canal.

In normal conditions the salinity of the GBL is influenced by the time of residence of water over the bottom salt deposits, the exchange of water with the southern and northern parts of the canal, and evaporation and meteorological conditions in the region. But in the special situation after the Suez Canal was closed, the first two factors were the principal causes of the exceptionally high salinity of the GBL and the formation of a brine layer near its bottom. The rate of salinity increase is directly proportional to the time of residence of water over the salt bed and inversely proportional to the rate of water flow through the Suez Canal. This was confirmed by observations[7] which show that during June–July, when the northward flow in Suez Canal becomes very slow and thus the time of residence becomes relatively longer, the salinity of the GBL reaches its maximum value of the year.

Table 1 Variation of salinity and temperature with depth at a station in the GBL (Fig. 1) occupied on June 20, 1974

Depth (m)	Salinity (‰)	Temperature (°C)
0	48.50	30.32
6	63.60	28.41
12	151.29	26.64
14	311.17	25.90
16	314.93	25.63

Table 2 Salinity–depth profile at stations 10, 11 and 12 in the GBL observed on April 5, 1967 (ref. 8)

Depth (m)	Salinity °/oo		
	10	11	12
0	43.23	43.41	43.63
3	43.48	43.75	43.93
6	43.88	43.93	44.02
9	43.97	44.04	—
12	44.35	—	—

After June 1967 the Suez Canal was closed to navigation and many obstacles and wrecks were blocking the waterway. Moreover, during the period from October 1973 to June 1974 the Canal was completely blocked by an earth dam at Deversoir where the Canal enters the northern part of the GBL. So the normal exchange of water between the Red and Mediterranean Seas through the Suez Canal was very much reduced and eventually stopped. This exchange was the chief cause for the gradual decrease of the salinity of the GBL from 168.8‰, during its filling process, to an average of 46‰ in April 1967. As the water passes over the salt bed, a considerable quantity of salt goes into solution and is eventually carried away by the water flow to the adjacent seas. In the new situation the GBL was almost isolated and its water became stationary over the salt bed allowing the salt dissolution to continue for a time long enough to bring the bottom water very close to its saturation point. The strong stratification and the great stability of the water column observed in June 1974 (Table 1) overcame the effects of wind stirring and evaporation.

The dense water (density about $1.2\,\mathrm{g\,ml^{-1}}$) near the bottom of the GBL acted as a barrier to divers, who found it difficult to penetrate. The black mud on the top of the salt bed was also observed by the Cambridge Expedition[4] in 1924 and in 1966–67 by El-Sharkawy[8].

It seems likely that as soon as the exchange between the canal and the adjacent seas returns to its normal magnitude, the brine layer will disappear and the salinity at all depths will decrease. I estimate that there will be a sharp drop in the salinity of the brine layer in the first few years followed by a gradual slow decrease afterwards. This assumption is based on the fact that after opening the Suez Canal in 1869 the average annual decrease of the salinity of the GBL was 0.36‰ in the period from 1872 to 1898, became 0.27‰ from 1898 to 1924 and then 0.13‰ from 1924 to 1966 (ref. 8). Although there is no record of the rate of decrease of the salinity in the period from June 1869, during the filling process when the salinity was 168.8‰, to 1872, this rate can be approximately estimated. Taking the salinity in 1966 as 46‰ and the salinity decrease in the period from 1872 through 1966 as 21.84‰, it follows that the salinity in 1872 was of the order of 67.8‰. This means that the annual decrease of salinity in the first two years after opening the canal might have been very sharp.

I strongly suggest that a thorough investigation of the physical, chemical and biological characteristics of the Bitter Lakes and Suez Canal could be made during the present transitional period.

AMIN H. MESHAL*

*Institute of Oceanography and Fisheries,
Alexandria, Egypt*

*Present Address: Air–Sea Interaction, Department of the Environment (AOL), Bedford Institute of Oceanography, Dartmouth, Nova Scotia, Canada B2Y 4A2

1 *Knudsen Hydrographical Tables* (G. E. C. Gad, Copenhagen, 1901).
2 Morcos, S. A., and Riley, J. P., *Deep Sea Res.*, 13, 741–749 (1966).
3 Morcos, S. A., *Proc. R. Soc. Edinb.*, B72, 449–458 (1972).
4 Fox, M. H., *Trans. zool. Soc. Lond.*, 22, 1–64 (1926).

5 Wust, G., *Naturwissenschaften*, 22, 446–450 (1934).
6 Faouzi, H., *Ann. Centre Univ. Hediter*, 5, 23–30 (1951).
7 Morcos, S. A., and Messieh, S N., *Limnol. Oceanogr.*, 18, 121–130 (1973).
8 El-Sharkawy, S M., thesis, Univ. Cairo (1969)

Confinement of thermonuclear microexplosions in imploding vortices

PRESENT efforts towards laser induced fusion envision spherical pellets consisting of the thermonuclear material in the centre, surrounded by a shell of some inert pusher material and an outer ablator shell. The same principles for the pellet design can be used if the implosion process is induced by a relativistic electron beam or intense ion beam (ref. 1, papers presented to Fifth IAEA Conference, Tokyo, 1974 and J. W. Shearer, unpublished). At least one other design of a non-spherical fuel pellet has been published[2]. This is non-symmetric and would have the advantage of using just one laser beam for target bombardment. But in all of these designs there remains one principal difficulty: laser, electron or ion beams with a sufficiently large total energy output for high density pellet compression and efficient thermonuclear burn have a comparatively long pulse length. Therefore, in order to use these beams for the required high density pellet compression rather large aspect ratios $r/\Delta r$ (r=pellet radius, Δr=thickness of pusher and ablator material) are dictated. These may result in rapidly growing Taylor-type instabilities. The growth rate for these instabilities could in principle be reduced below dangerous levels by making the pellet and implosion process highly symmetrical. But, since the symmetry requirements are extreme this may be difficult to achieve. The growth rate for the Taylor instabilities could also be reduced by smaller aspect ratios, but smaller aspect ratios need shorter and precisely shaped pulses, which again are difficult to achieve.

I propose here an alternative pellet design which assures Taylor-stable implosion at high aspect ratios. Figure 1 shows the new pellet design in two cross sections. The pellet has the form of a circular cylinder with cylindrical liners of spiral shape embedded in the pusher and ablator material. The arrows indicate the beams radially and axially bombarding the pellet. In addition to the radial beams, two axial beams are required to compress the thermonuclear material within the pellet axially. The presence of the spiral liners, which are made of some heavy inert material, results in an azimuthal velocity component for the ablated material. If the liners have the form of logarithmic spirals, the ratio of the radial to the azimuthal ablation velocity is constant during the ablation process. As a result of the radial ablation the pellet material is imploded. But the implosion process is here governed not only by the conservation of radial linear momentum but also by the conservation of angular momentum. This results in a collapsing vortex similar to a tornado funnel. The azimuthal motion in the collapsing vortex will give rise to two effects. First, it will stop

Fig. 1 The new pellet design. *a*, Plan; *b*, elevation.
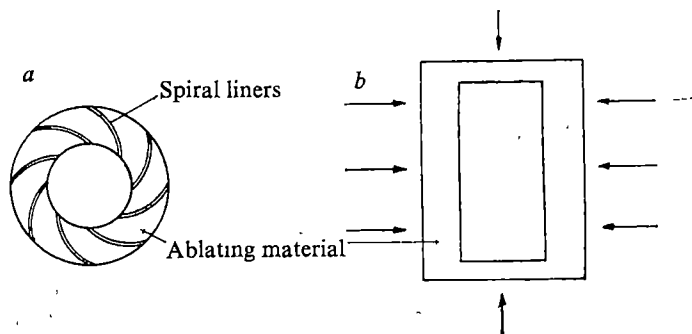
the collapse at a minimum radius of the vortex core at the moment the centrifugal forces become equal to the implosion stagnation force; second, the rapid rise of the centrifugal force during the collapse will make the implosion process Taylor-stable (as in the case of the formation of a tornado funnel). The ratio of collapse (r_0/r_1, where r_0 is the initial radius of the vortex core and r_1 its final minimum radius) can be predetermined by a proper choice of ratio $v_r^{(0)}/v_\varphi^{(0)}$ for the initial ratio of the radial to the azimuthal implosion velocity and thus the slope of the liner spirals. At maximum collapse $v_r = v_r^{(1)} = 0$ and $v_\varphi = v_\varphi^{(1)}$.

Conservation of energy and angular momentum then require that

$$(v_\varphi^{(1)})^2 = (v_r^{(0)})^2 + (v_\varphi^{(0)})^2 \qquad (1)$$

$$r_1 v_\varphi^{(1)} = r_0 v_\varphi^{(0)} \qquad (2)$$

From this one obtains

$$v_r^{(0)}/v_\varphi^{(0)} = [(r_0/r_1)^2 - 1]^{1/2} \simeq r_0/r_1, \quad r_0 \gg r_1 \qquad (3)$$

If one assumes a tenfold compression in radius this yields $v_r^{(0)}/v_\varphi^{(0)} \simeq 10$.

An inertial force directed towards decreasing density results in a Taylor-unstable situation and one directed towards increasing density results in Taylor-stable fluid flow. For the case in question the inertial force resulting from the radial deceleration

$$a_1 \simeq (v_r^{(0)})^2/r_0 \qquad (4)$$

is destabilising, whereas the centrifugal force resulting from the acceleration

$$a_2 \simeq (v_\varphi^{(1)})^2/r_1 \qquad (5)$$

is stabilising. Stability is guaranteed if $a_2/a_1 \gg 1$. From equations (2) and (3) this condition leads to

$$a_2/a_1 \simeq r_0/r_1 \gg 1 \qquad (6)$$

which in my chosen example, $r_0/r_1 = 10$, is well satisfied.

The proposed scheme of a collapsing vortex may be used also for the stable confinement of a microexplosion induced by a high density gas-embedded pinch. Because of the longer time scale involved in such a case, the ablator material may be made out of high explosive material which is radially detonated, eliminating the need for laser or other intense beams to affect the ablation-induced implosion and compression.

F. WINTERBERG

Desert Research Institute,
University of Nevada System,
Reno, Nevada 89507

1 Winterberg, F., *Nature*, **251**, 44 (1974); *Plasma Physics*, **17**, 69 (1975).
2 McCall, G. H., and Morse, R. L., *Laser Focus*, **12**, 40 (1974).

Formation of blisters in molybdenum bombarded with helium

THE formation of surface blisters on metals bombarded with low energy inert gas ions was first observed by Primak[1] but only recently, when the potential importance of blistering in the first wall of a fusion reactor became recognised, has much further attention been paid to the phenomenon[2,3]. Most of this work has used scanning electron microscopy to examine the blistered surface although considerable information has also been obtained from gas release measurements both during bombardment and in post-bombardment anneals. Transmission electron microscopy has been used to a lesser extent but nevertheless has yielded important information on the substructure within the layer where the gas atoms came to rest—normally some hundreds of Ångstroms from the metal surface. For example, several workers have shown that gas bubbles form in thin foils irradiated with low energy (<300 keV) helium or argon ions[4–6]. One interesting fact (D. J. Mazey, unpublished) is that over a wide temperature range from 600 °C down to 20 °C, 36 keV helium bombardment of molybdenum produces a fine distribution of helium bubbles crystallising on to a body-centred-cubic (b.c.c.) lattice analogous to the void lattice[4,7]. Here I describe an extension of transmission microscopy to view the blisters themselves and suggest some details of the blister formation mechanism.

Molybdenum samples were irradiated at room temperature with 5×10^{17} 18-keV helium ions per cm^2, these ions having a projected range of approximately 630 Å. Following bombardment the samples were electropolished using a backthinning technique to protect the front damaged surface so that this surface was contained in the final electron-transparent thin section. Both a Philips EM 300 100 keV electron microscope and the Harwell AEI High Voltage Microscope were used for specimen examination. On either microscope the presence of the blisters (Fig. 1),
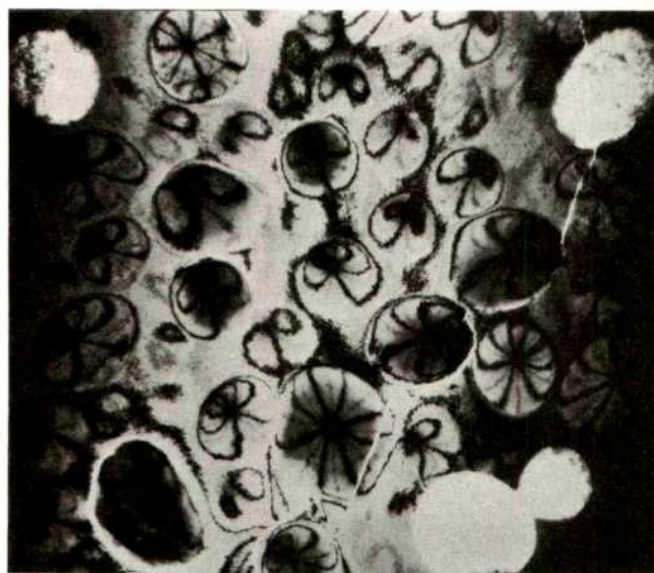
Fig. 1 800-keV electron micrograph of blisters in helium-bombarded molybdenum. The blisters have diameters between 1 and 1.5 µm.

was made very obvious by the bend contours associated with their dome-shaped profile. The contour patterns seem to be a good example of the type used by Steeds *et al.*[8] for real space crystallography; it was simple to index each contour and use the poles (Fig. 2) to measure the blister radius of curvature. On a finer scale the damage structure consisted of a high density of dislocations—thus making the bend contours considerably less sharp than in a perfect crystal—and a very high concentration of small helium bubbles, radius 15–20 Å. These bubbles were in both the unblistered areas and the blister covers.

McCracken[9] has briefly outlined the process of blister formation as the coalescence of small helium bubbles to form a large high pressure bubble sufficient to deform the surface. This seems to be consistent with the experimental
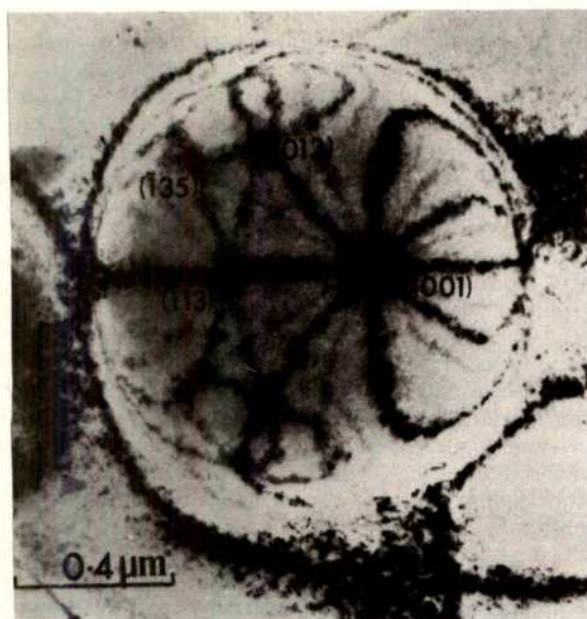
Fig. 2 A more detailed micrograph of blister bend contours with the main poles indexed.

observations but any detailed mechanism besides describing the formation of the blisters from the small bubbles must also explain at least two important results in the blistering work:

(1) Both scanning electron microscopy and gas release results have demonstrated that a relatively sharp threshold dose exists for blister formation.

(2) At high temperatures the bombarded surface displays a 'pinhole' structure rather than blisters[5,10].

The possible mechanism suggested here is based on the assumption that the coalescence of growing bubbles (at temperatures below that at which bubbles can thermally migrate) only becomes rapid when the bubbles are essentially touching—in other words when the volume swelling due to the bubbles reaches a critical value. In the case of the bubbles being on a perfect b.c.c. lattice, it is easy to show that the volume swelling ($\Delta V/V$) would be 68% but in the random array the situation is a little less certain. But if we proceed on this basis, the reason for the existence of a critical dose is evident and is governed by the maximum volume swelling that can be supported in the peak of the helium stopping distribution. The interesting point is that because the threshold condition is a volume one, then coalescence without any decrease in volume—the likely situation unless the bubble can quickly acquire excess interstitials—will not relieve the tendency for further coalescence. So, on this model the coalescence, once started, becomes a runaway process. From the point of view of blistering the important thing is that the coalescence of two gas bubbles at constant volume must increase the excess internal pressure. For example, if we take two equal equilibrium gas bubbles, radius r, then the internal pressure, p, is given by $p = 2\gamma/r$ where γ is the surface energy. On coalescing, the new equilibrium gas pressure will be lower by a factor of $2^{\frac{1}{3}}$, giving the bubble an excess internal pressure 26% above the equilibrium. As further coalescence takes place, this value will rapidly increase until enough excess pressure is built up to deform the metal surface and create a blister. For the small gas bubbles observed, the equilibrium gas pressures are in the region of 10^4 atmospheres.

At high temperatures the availability of thermal vacancies becomes important[11] between 0.4 and $0.5T_m$. It is therefore possible to define a temperature where the coalesced bubbles immediately collect enough vacancies to relieve their excess internal pressure and regain equilibrium. In this way, not enough pressure can build up to deform the metal surface and blisters will not be formed. Instead the bubbles grow until they cut the surface thus producing the observed 'pinhole' effect.

One important prediction of this model is that in the fusion reactor first wall, which will have a spectrum of ion energies, the integrated dose necessary to build up the critical helium concentrations at any particular depth will be far greater than those found experimentally with mono-energetic beams. Such results would overestimate predicted first wall surface erosion rates and thus the detrimental effects of blistering could be less than anticipated.

Metallurgy Division,
Atomic Energy Research Establishment,
Harwell, UK

1 Primak, W., *J. appl. Phys.*, **34**, 3630 (1963).
2 *Applications of Ion Beams to Metals* (edit. by Picraux, S. T., EerNisse, E. P., and Vook, F. L.), (Plenum, New York, 1974).
3 *Proc. Conf. on Surface Effects in Controlled Fusion Devices, J. nucl. Mat.*, **53** (1974).
4 Sass, S. L., and Eyre, B. L., *Phil. Mag.*, **27**, 1447 (1973).
5 Thomas, G. J., and Bauer, W., in *Applications of Ion Beams to Metals* (edit. by. Picraux, S. T., EerNisse, E. P., and Vook, F. L.), 533 (Plenum, New York, 1974)
6 Hertel, B., Diehl, J., Gotthardt, R. and Sultze, H., *ibid.*, 507.
7 Evans, J. H., *Nature*, **229**, 403 (1971).
8 Steeds, J. W., Tatlock, G. J., and Hampson, J., *Nature*, **241**, 435 (1973).
9 McCracken, G. M., *Rep. Progr. Phys.*, **38**, 241 (1975).
10 Erents, S. K., and McCracken, G. M., *Rad. Effects*, **18**, 191 (1973).
11 Martin, D. G., *Culham Report*, R-103 (UKAEA, 1970).

A novel method for the calculation of energies of activation for gas reactions

A GENERAL method for the rapid, accurate calculation of activation energies for gas reactions would clearly be of great value. To date, full *ab initio* quantum mechanical calculations of reaction coordinates and potential energy surfaces have been carried out for only the simplest systems and extensions to larger ones are still some way off[1]. On the other hand, semi-empirical approaches[2] which could perhaps deal with larger species require a good deal of accessory experimental information.

We suggest here a simple new approach, based on self-consistent molecular orbital calculations of the Pople–Segal–Santry type[3,4]. This leads first to the heat of activation, $\Delta H^{\ddagger}$ for a reaction and through this to a value for the energy of activation ($\Delta E^{\ddagger}$).

The use of the 'bond index' has been derived and illustrated previously[5,6]. This quantity stems from the density matrix which is, in turn, readily obtainable from a self-consistent field molecular calculation. It is found, for example, that, in the case of CH_4, the C–H bond index is 0.987, and the C–C bond indices in the series C_2H_6, C_2H_4, C_2H_2 are 1.023, 2.032, 2.989. These indices clearly correspond to the chemist's concept of a 'single' bond or a 'double' bond and, on this basis, we suggest that the bond indices can be used, together with experimental heats of atomisation in the standard state, to generate an improved set of standard energy terms for a variety of bonds. These will then allow prediction of the heats of formation for molecules containing them. A particular improvement over the present bond energy concept is that the bond index reflects slight changes in intrinsic bond strength with the environment of the bond in question, further details of which will be published elsewhere (P.G.P.). But an important extension arises because bond indices can be calculated for any system, and can thus

afford, in principle, the heat of atomisation for that system in the standard state.

So the method should be applicable to a species undergoing reaction. By constructing a grid of points on a surface each corresponding to a particular geometry we can follow a reaction path over a saddle. Because of the way in which the bond energy terms are initially defined, we thus obtain the heat of activation, $\Delta H\ddagger$, in the standard state. Calculation of the energy of activation, $\Delta E\ddagger$, follows in a standard way[7].

We here apply the technique to two related hydrogen-transfer reactions, namely, hydrogen abstraction from methane by the methyl and the trifluoromethyl radical,

$$
\begin{array}{ccc}
CH_3\cdot & & \nearrow CH_4 \\
+ \ CH_4 & & \\
CF_3\cdot & & \searrow CF_3H
\end{array}
\quad + \ CH_3\cdot
$$
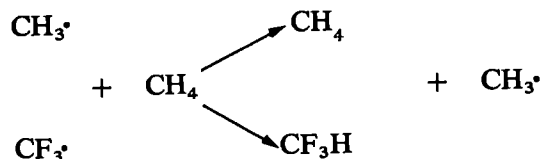
We assumed that all positions on the reaction coordinate have C_{3v} symmetry, thus,

$$ -\overset{\diagup}{\underset{\diagdown}{C}} - H - \overset{\diagup}{\underset{\diagdown}{C}} - $$
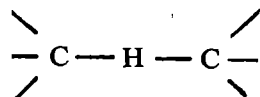
We further assumed that, in the reaction between methyl and methane, the starting and resultant methyl radicals undergo linear changes in the planar $\longleftrightarrow$ pyramidal angles. The $CF_3\cdot$ radical was assumed pyramidal throughout. At the starting point the reactants were separated by 0.5 nm. An energy search was carried out around the transition state in each case and the reaction coordinates were constructed. The activation energies, corrected for specific heat changes on formation of the transition

Table 1 Energies of activation for the reactions $CH_3\cdot$ or $CF_3\cdot +CH_4 \rightarrow CH_4$ or $CF_3H+CH\cdot$

Reactant	$\Delta E\ddagger$ (kJ mol^{-1})	
	Observed	Calculated
CH_3	59.0, 59.8, 62.3*	56.2
CF_3	46.0†	49.4

*Tables of Bimolecular Gas Reactions, A. F. Trotman-Dickenson and G. S. Milne.
†As for *; recommended value.

state, are given in Table 1. These are compared with the experimental values by assuming a temperature in the middle of the experimental range. The agreement is very encouraging and we are now investigating the use of the approach on a series of other gas reactions.

THOMAS N. BELL

Department of Chemistry,
Simon Fraser University,
Burnaby 2, British Columbia, Canada

PETER G. PERKINS

Department of Pure and Applied Chemistry,
University of Strathclyde,
Glasgow G1 1XL, UK

1 Laidler, K. J., *Theories of Chemical Reaction Rates*, 8 (McGraw Hill, New York and London, 1969)
2 Johnston, H. S., *Gas-Phase Reaction Rate Theory*, 177–183 (Ronald Press, New York, 1966).
3 Pople, J. A., Santry, D. P , and Segal, G. A., *J. chem. Phys*, 43, S129 (1965)
4 Armstrong, D. R., Perkins, P. G., and Stewart, J. J. P , *J. chem. Soc. A*, 3654 (1971).
5 Armstrong, D. R., Perkins, P. G., and Stewart, J. J. P., *J. chem. Soc., Dalton Trans.*, 838 (1973)
6 Armstrong, D. R., Perkins, P. G., and Stewart, J J. P., *J. chem. Soc., Dalton Trans.*, 2273 (1973).
7 Glasstone, S., Laidler, K. J., and Eyring, H., *The Theory of Rate Processes*, ch. IV (McGraw-Hill, New York and London, 1941).

Stereochemistry of some steranes from geological sources

THE steranes present in crude oils and sedimentary rocks are believed to be derived from the sterols of ancient organisms. Their precise structure and stereochemistry may give important clues about the nature of the original biological materials and about the geochemical processes which they have undergone. Examination of synthetic steranes[1,2] has led us to question the adequacy of reported[3-6] identifications, using only gas–liquid chromatography (GC) and mass spectroscopy (MS), of steranes such as 5α-cholestane, 5α-ergostane, and 5α-stigmastane (=5α-sitostane) in geological samples. The last two, in particular, seem incompletely identified, since the configuration at C-24 has been ignored (although it is of phylogenetic importance in modern organisms[7,8]), and since we find that epimers at C-24, that is II and III, IV and V, are indistinguishable using GC and MS. The use of the names 5α-ergostane and 5α-stigmastane in geochemical work both for steranes of undetermined configuration at C-24 and for synthetic samples of defined configuration is confusing. In the absence of clear IUPAC/IUB rules, we have adopted the names shown in Fig. 1.

The structure and absolute stereochemistry of many steranes can, however, be defined[1,2]; after isolation, by the additional use of optical rotatory dispersion (ORD) and high-resolution (100 or 220 MHz) proton magnetic resonance (PMR) spectroscopy. Preliminary results on the application of these methods to steranes from two crude oils and from Green River Shale are reported here.

A crude oil of unknown age from Rozel Point, Utah[9], was distilled, chromatographed on silicic acid and alumina, and treated with urea to give a sterane-rich fraction. Examination using GC and MS revealed a large number of C_nH_{2n-6} ($n = 26$–30) components with sterane-type fragmentation patterns.

Further separation was achieved by thiourea adduction and preparative GC. A component having the retention time of 5α-cholestane was recrystallised from propanol and confirmed as 5α-cholestane (I) by melting point (80–80.5 °C), mixed melting point, MS, PMR, and ORD. A second recrystallised isolate (melting point 80.5–81 °C), indistinguishable from 5α-ergostane using GC and MS, was revealed as a 1:1 mixture of 5α-campestane (II) and 5α-ergostane (III) using PMR and ORD. These two steranes were separated on alumina (2.8 m × 5 mm column eluted with pentane; method to be published) and the identity of each was confirmed using PMR spectroscopy. The presence in the thiourea adduct of (24R)- and (24S)-4α, 24-dimethyl-5α-cholestanes in a similar ratio was also demonstrated. A component having the GC and MS properties of 5α-stigmastane was isolated from the thiourea non-adducted material and examined without recrystallisation; PMR analysis indicated a 1:1 mixture of 5α-stigmastane (IV) and 5α-poriferastane (V), consistent with the ORD spectrum.

Similar fractionation of a crude oil of Upper Miocene origin from Huntington Beach, California[10,11] also yielded a 1:1 mixture of the epimers II and III. By contrast, the corresponding fraction isolated from a sample of Green River Shale (Eocene) from the Rifle Mine, Colorado, proved to be a 25:75 mixture of epimers II and III, together with a small quantity of an unidentified third component, whereas the major C_{28}-sterane fraction proved to be a 30:70 mixture of IV and V. Although comparisons between data from different samples of the vast
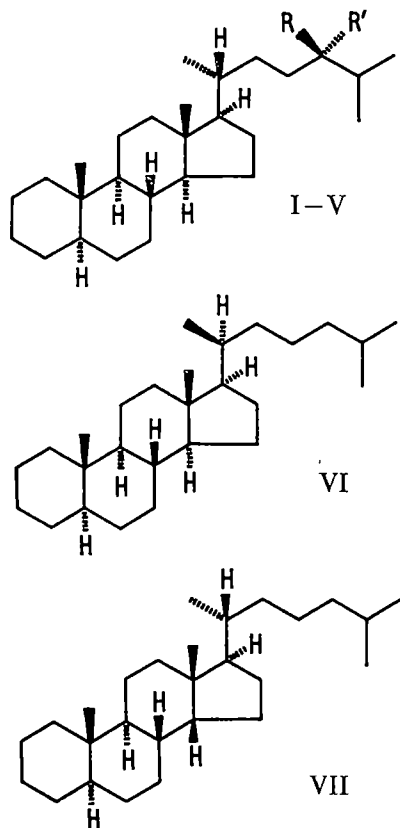
Fig. 1 Structural formulae of steranes isolated from geological sources. I: R = R′ = H; 5α-cholestane. II: R = methyl group; R′ = H; 5α-campestane (24R). III: R = H; R′ = methyl group; 5α-ergostane (24S). IV: R = ethyl group; R′ = H; 5α-stigmastane (24R). V: R = H; R′ = ethyl group; 5α-poriferastane (24S)

Green River Shale formation require caution, our result for the C_{28}-sterane is compatible with the published ^{13}C nuclear magnetic resonance spectrum[12] of Green River Shale "5α-ergostane", which shows deviations from the reference spectrum in the regions (161 and 174 p.p.m. from CS_2) where we find 5α-campestane (II) to differ from 5α-ergostane (III). This is consistent with the presence of a significant proportion of II in that sample.

At least two stereoisomers of 5α-cholestane are present in oil from Rozel Point. (20S)-5α-cholestane (VI) was isolated from the thiourea non-adduct and identified by comparison (GC, MS, PMR, ORD) with an authentic sample[2]; its concentration in the crude oil seems to be comparable with that of 5α-cholestane. A minor C-27 component of the thiourea adduct, homogeneous by GC analysis on several stationary phases, had the retention time of 5α,14β-cholestane (VIII), (ref. 2) and a similar mass spectrum, although the optical activity was considerably lower than expected. The PMR spectrum was interpreted as that of a 40:60 mixture of VII and an isomer in which the 18-CH_3 and 21-CH_3 signals are shifted upfield (δ 1.05 and 0.865, respectively, in C_6D_6). A possible structure for the latter is (20S)-5α,14β-cholestane, and the synthesis of a reference sample is in hand.

Although investigation of these geological samples is incomplete (for example, the identified steranes account for <20% weight for weight of all sterane-like hydrocarbons in Rozel Point crude oil), several interesting points are raised. The preponderance in Green River Shale of one epimer of each of the 24-alkyl-5α-cholestanes suggests that in relatively mild conditions of diagenesis the stereochemistry of precursor sterols is at least partly preserved in the hydrocarbon products; similarly, pristane from this source largely retains the stereochemistry of its putative precursor[13]. The predominant (24S)

stereochemistry may, in addition, point to possible source organisms such as algae[8,14]. The phylogenetic significance of the stereochemistry at C-24 requires further study, however, especially as it is now known that some higher plants produce 24-methylsterols of both (R) and (S) configuration (Mulheirn, L. J., unpublished). Our results also suggest that equilibration at C-20 and C-24 may prove characteristic of petroleum steranes, reflecting the more severe conditions required for petroleum formation. No stereochemical analyses of phytane or pristane from crude oils have been reported, although it may be relevant that farnesane from New Ulm (Texas) crude exhibits complete randomisation of stereochemistry at its two centres of asymmetry (J. W. Cornforth and J. R. Maxwell, personal communication). The occurrence of minor amounts of a 14β-sterane in Rozel Point oil may also be attributed to an equilibration process, the scanty available evidence[15] suggesting that this configuration is the less stable. No natural sterols with a 14β-hydrogen are known.

Finally, the data demonstrate for the first time that the isolated steranes possess the same absolute stereochemistry as that of the corresponding hydrocarbons synthesised from the sterols of modern organisms. No optical rotations have previously been reported for individual steranes from geological samples.

We thank Professor J. W. Cornforth for initiating this work; Drs A. Hood and P. R. Mommessin (Shell Development Company, Houston, Texas) for arranging the supply of the oils and shale; our colleagues at Shell Research Ltd, Thornton Research Centre, Cheshire for preliminary processing of the oils; and Mrs D. Davis, Mrs P. R. Jennings and Mrs S. A. Terry for technical assistance.

L. J. MULHEIRN
G. RYBACK

Shell Research Limited,
Milstead Laboratory,
Sittingbourne Research Centre,
Sittingbourne, Kent, ME9 8AG, UK

1 Mulheirn, L. J., *Tetrahedron Lett*, 3175–3178 (1973)
2 Mulheirn, L J , and Ryback, G , *J. chem. Soc Chem Comm*, 886–887 (1974)
3 Hills, I. R., Smith, G. W , and Whitehead, E. V , *J. Instn Petrol*, 56, 127–137 (1970).
4 Ikan, R., and Bortinger, A., *Israel J. Chem.*, 9, 679–682 (1971)
5 Gallegos, E J , *Analyt Chem.*, 43, 1151–1160 (1971)
6 Maxwell, J R., Pillinger, C T., and Eglinton, G , *Q Rev chem Soc.*, 25, 571–628 (1971).
7 Goad, L. J., and Goodwin, T. W , *Prog. Phytochem.*, 3, 113–198 (1972).
8 Nes, W. R , *Lipids*, 9, 596–612 (1974).
9 Eardley, A. J , *Spec Stud. Utah geol. mineral Surv*, 5 (1963)
10 Hoering, T C , *Yb Carnegie Instn Wash*, 303–307 (1969)
11 Philippi, G. T , *Geochim cosmochim Acta*, 29, 1021–1049 (1965).
12 Balogh, B , Wilson, D. M , and Burlingame, A. L , *Nature*, 233, 261–263 (1971)
13 Maxwell, J. R., Cox, R E., Ackman, R. G , and Hooper, S. N , in *Advances in Organic Geochemistry 1971* (edit by von Gaertner, H. R , and Wehner, H.), 277–291 (Pergamon, Oxford, 1972)
14 Bradley, W H , *Bull. geol Soc Am.*, 81, 985–1000 (1970)
15 Van Horn, A. R , and Djerassi, C , *J. Am. chem Soc*, 89, 651–664 (1967).

Influence of interannual climatic fluctuations on biological systems

GROWTH of conifers in western North America and the distribution of albacore tuna (*Thunnus alalunga*) along the west coast of North America are linked by large scale atmospheric flow patterns which are influenced by air–sea interaction processes over the eastern North Pacific Although the systems respond to their respective environments during different times of the year there is strong evidence that they are reacting to the same climatic fluctuations.

Ring widths of conifers from a variety of semi-arid sites in western North America reflect climatic variations. The spatial anomaly patterns of tree growth have been related to large scale anomaly patterns of the general atmospheric circulation which, in turn, are related to anomaly patterns

of sea-surface temperature (see, for example, refs 1 and 2).

Seasonal migrations of albacore tuna into and out of the coastal North American fishery and the population distribution along the coast are related to changes in the thermal structure of the eastern North Pacific which, in turn, are related to air–sea interaction events[3].

Although it would be desirable to know the yearly variations in the distribution of albacore abundance or the exact number of individuals in the population, the best source of available data is landing statistics compiled for various regions along the North American coast (J. A. Renner, personal communication). These data indicate albacore availability, or accessibility to the efforts of a fishery. A complex situation probably exists where fluctuations in landings result both from environmental influences affecting albacore migration routes (causing yearly variations in their geographical distribution along the coast) and from weather conditions affecting the location and success of the fishermen and/or the behaviour of the fish once they are distributed.

As an indicator of variations in albacore population distribution, the landings reported north of San Francisco were expressed as a percentage of those reported along the entire coast. This assumes the fishing effort is spread along the entire coast throughout each fishing season. Although a complete effort analysis has not been made, boats were operating consistently along the entire coast throughout the analysis period.

To determine whether spatial patterns of tree growth

from 49 sites in western North America and albacore distribution both respond to the same variations in the ocean-atmosphere system, the large scale components of the ring-width variance were transformed into uncorrelated variables (amplitudes of principal component eigenvectors) and then put in stepwise regression to find which of these amplitudes were significant predictors (at the 95% confidence level) of albacore catch. This procedure gave the equation:

$$Y = 35.12 + 5.76A_2 + 5.13A_8 + 8.56A'_4 - 6.17A'_9 + 11.54A'_{10} \quad (1)$$

where Y is the estimated percentage of albacore caught north of San Francisco, A and A' are eigenvector amplitudes corresponding to the catch year and the following year, respectively, and subscripts refer to the eigenvector number (Fig. 1). The regression accounted for 83% of the catch data variance in the calibration period (Fig. 2). The values of tree-growth eigenvector amplitudes for 1700 to 1938 were then substituted into equation (1) to estimate what the yearly albacore catch distribution would have been for this early period based on the patterns of tree growth (Fig. 3).

Although fishery statistics are available after 1961, tree-growth data for all 49 sites within the spatial network are not, and the only independent data available to check the validity of the reconstruction are incomplete fishery catch statistics between 1904 and 1937 (ref. 4). A qualitative comparison between both sets of data shows these common

Fig. 1 Maps of the first 10 eigenvectors of ring-width index. The percentage figures refer to the variance accounted for by the corresponding eigenvector. Numbered circles indicate ring-width chronology sites.
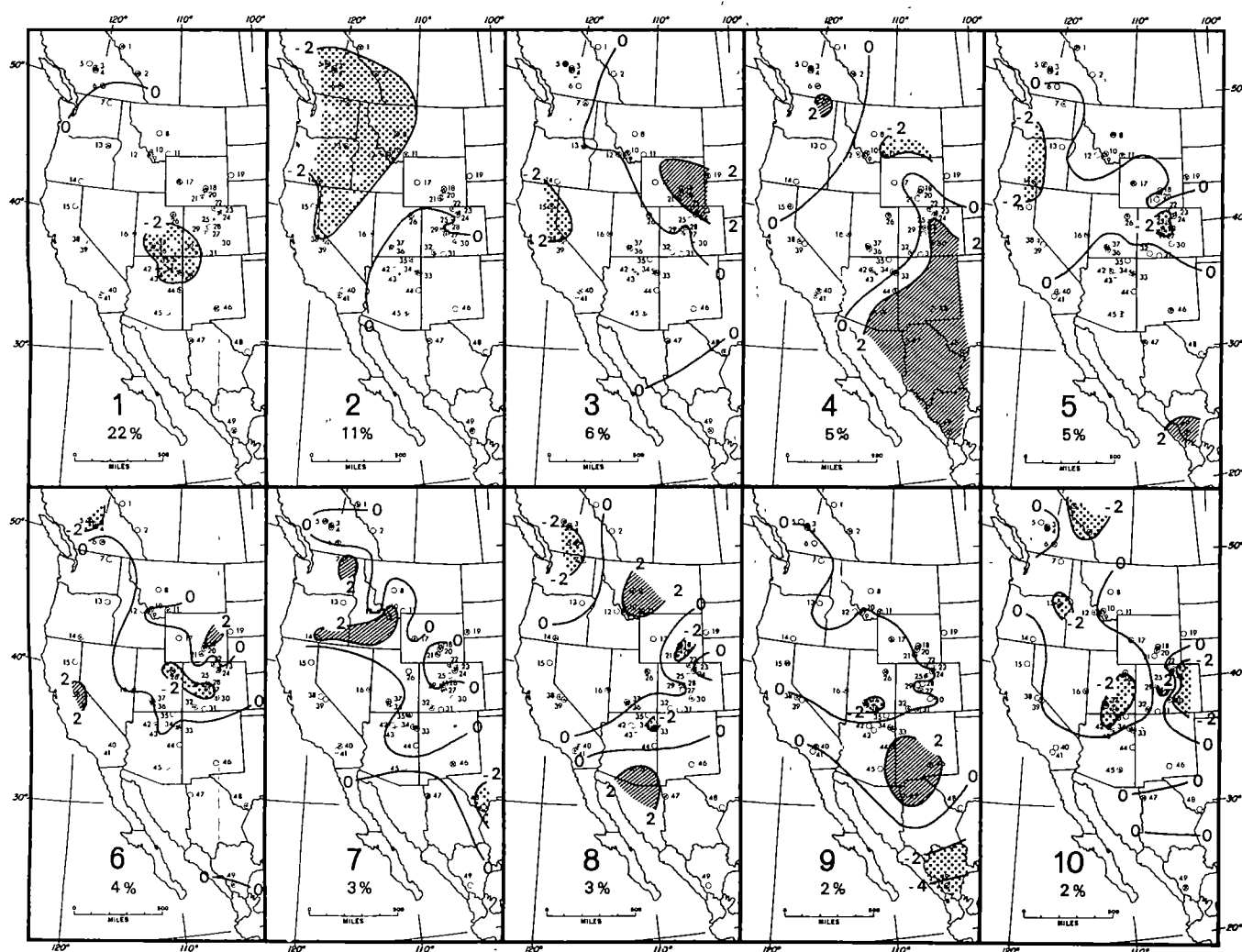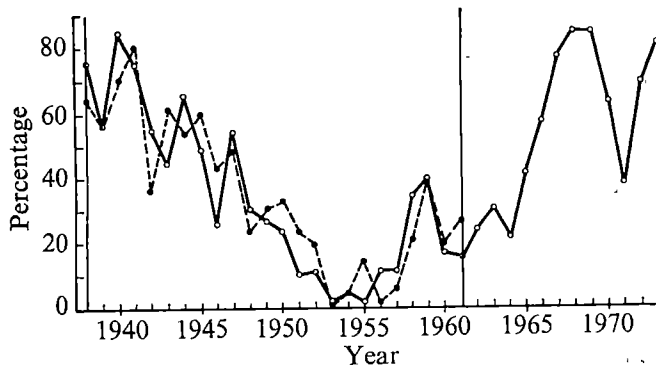
Fig. 2 Percentage of the total North American west coast albacore tuna catch taken north of San Francisco, estimated from landings data (———) and percentage catch taken north of San Francisco derived from the calibration equation applied to tree-growth data over the dependent data period (- - - - -). Calibration included the following steps: (1) We computed the 49 by 49 correlation matrix which expressed the interrelationships among the 49 ring-width index series for the period of analysis (1700–1962). (2) The eigenvectors of the correlation matrix were then computed. These (orthogonal) eigenvectors may be expressed in map form as characteristic spatial anomaly patterns of tree growth. The 10 eigenvector patterns which accounted for the most variance (the large scale variance) were selected for further analysis. Maps of these eigenvectors are shown in Fig. 1. Linear combinations of these 10 eigenvectors can reproduce 64% of the ring-width variance for the 49 chronologies during the period of analysis. (3) Each of the first 10 eigenvectors was multiplied by the normalised tree-growth data at each site for each year from 1700 to 1962. The resulting matrix product consists of 10 time series of amplitudes which give the relative importance of each of the first 10 eigenvector patterns (the large scale patterns) in each year's tree-growth anomaly map. Being orthogonal to (uncorrelated with) each other they are easy to use as predictor variables in multiple regression. (4) Since trees respond to the climate of both the concurrent season and previous seasons, a prediction model was constructed including the 10 amplitudes corresponding to the same year as the catch data and the 10 amplitudes corresponding to the year following the catch data, for a total of 20 possible predictor variables. (5) Stepwise multiple regression was used to find those amplitudes that were significant predictors (95% confidence level) of albacore catch distribution for the years 1938–61.
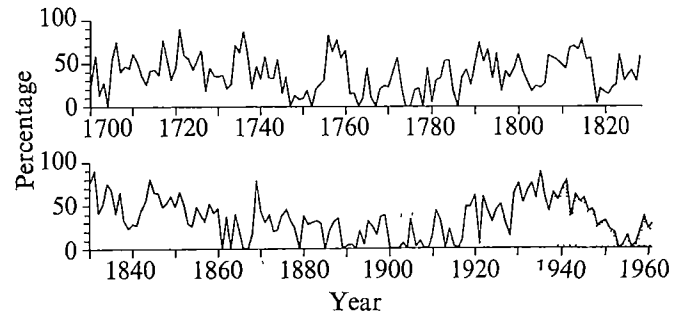
Fig. 3 Percentage of the total albacore tuna catch taken north of San Francisco, estimated from the calibration equation applied to tree-growth data for 1700 to 1961. Shading indicates calibration period.

characteristics· a fishery that was centred off southern California in the early 1900s; a sharp decrease in the availability of albacore to the southern fishery between 1917 and 1918; another decrease in availability of albacore to the southern fishery in 1923 when the total catch was the lowest since 1918; and a prolonged period of decreased availability to the southern fishing fleet after 1928. The reconstructed distribution data indicate that a fishery would have been centred quite far north after 1929 and through the late 1930s if boats had operated there during that period. The absence of albacore from the catch record for Oregon and Washington before 1937 and low catches for California after 1925 probably reflect the extreme northward and offshore population distribution indicated by the reconstructed data; the fish were just not available to salmon trollers that did operate close to shore north of California during this period[5].

We also examined the eigenvector patterns of tree growth that were chosen by regression to be associated with a high percentage of albacore caught north of San Francisco. The tree-growth pattern corresponding to the year of the catch
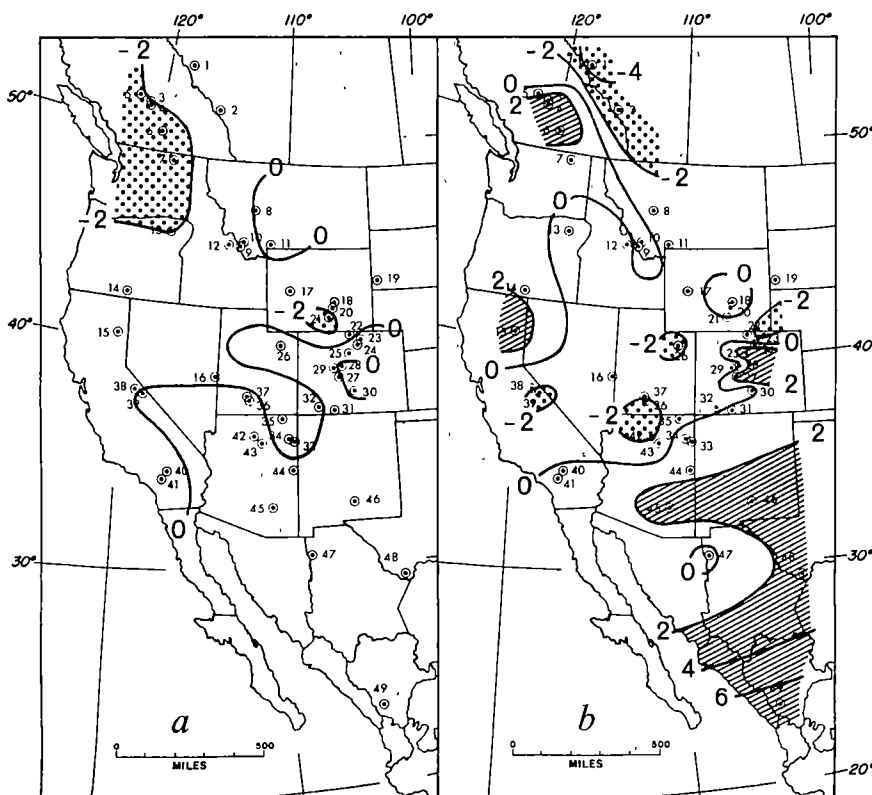
Fig. 4 Tree-growth anomaly patterns associated with above normal percentages of albacore caught north of San Francisco. Map *a*, corresponding to the year of the catch, is formed from the linear combination of eigenvectors 2 and 8 in equation (2); map *b*, corresponding to the year following the catch, is formed from the linear combination of eigenvectors 4, 9, and 10 in equation (3).

can be represented by a map composed of eigenvectors 2 and 8 as follows:

$$\text{map } a = 5.76 \text{ (eigenvector 2)} + 5.13 \text{ (eigenvector 8)} \qquad (2)$$

where the numerical coefficients are taken from equation (1). Similarly, the tree-growth anomaly pattern corresponding to the year following a high percentage of albacore caught north of San Francisco can be represented as:

$$\text{map } b = 8.56 \text{ (eigenvector 4)} - 6.17 \text{ (eigenvector 9)}$$
$$+ 11.54 \text{ eigenvector 10)} \qquad (3)$$

These maps are presented in Fig. 4. The ring-width data were mostly from trees sited in arid localities, so that a wide ring would generally be associated with anomalously cool, cloudy weather and above normal precipitation whereas a narrow ring would reflect warm, sunny and dry conditions.

Below, normal tree growth in the Pacific North-west (Fig. 4) is indicative of dry conditions associated with below normal cyclonic activity during the fishing season. Sunny and mild weather would favour albacore fishing in adjacent waters, as would above normal insolation regardless of weather. The resulting excess of stored heat in the ocean would be given up through evaporation during the following autumn and winter and lead to increased cyclonic activity and precipitation along the coast north of San Francisco. These conditions would lead to increased tree growth during the following growing season (Fig. 4).

Autumn and winter climatic anomaly features, combined with spring climate and the year-to-year autocorrelation of tree-ring widths, produce the other ring-width anomaly features in Fig. 4 for the following growing season. Narrow ring widths south of San Francisco, for example, imply below normal precipitation—an expected feature since winter precipitation in the Pacific North-west is negatively correlated with winter precipitation in southern California[6].

The reconstructed values of albacore catch distribution data (Fig. 3) and inferred population distribution also seem to exhibit long term changes over intervals of 100 yr or more, which suggest the possibility that long term fluctuations in the ocean–atmosphere system may be involved.

The success of the calibration of tree rings with albacore catch indicates the possibility of relating tree-ring variations to any type of biological variations which are affected by large scale climatic fluctuations. Such relationships may be quantified and used to reconstruct objectively other climatically-caused biotic variations in the past.

N. E. CLARK

National Oceanic and Atmospheric Administration,
National Marine Fisheries Service,
Southwest Fisheries Center,
La Jolla, California 92037

T. J BLASING
H. C. FRITTS

Laboratory of Tree-Ring Research,
University of Arizona, Tucson, Arizona 85721

1 LaMarche, V C, *Science*, 183, 1043–1048 (1974).
2 Namias, J, *Mon Weath. Rev*, U S *Dep. Agric*, 97, 173–192 (1969).
3 Laurs, R. M, *et al*, *Report of Joint National Marine Fisheries Service–American Fishermen's Research Foundation Albacore Studies Conducted During 1973* (National Marine Fisheries Service, Southwest Fisheries Center, La Jolla, 1973).
4 Clemens, H B, and Craig, W L., *Calif Dept Fish and Game, Fish Bull*, 128 (1965).
5 Sette, O. E, *Calif Coop Oceanic Fish Invest. Rept*, 7, 181–194 (1960).
6 Pyke, C B, *An Investigation of some precipitation patterns in California and adjacent regions* (University of California Water Resources Center, 1966).

Oats may grow better in water depleted in oxygen 18 and deuterium

WHILE growing oats at different temperatures in water of different ^{18}O and deuterium (D) abundances, we noticed that oats grown in Antarctic water in which is depleted in ^{18}O and D by $-49‰$ and $-400‰$, relative to standard mean ocean water (SMOW used as a comparative reference in hydrogen and oxygen isotope studies), showed initial growth 1–2 weeks sooner than did oats grown in water containing greater ^{18}O and D concentrations. The oats seemed to grow better in water which was most depleted in the stable isotopes throughout the growth period.

The oats were grown from the same batch of seeds in two sealed glass-covered glass jars (approximately 10 l). Twenty-five oat seeds were added to each jar, containing the same amount of vermiculite and 500 ml water to which 5.0 g Rapid-Gro, a commercial fertiliser, had been added. One jar contained melted glacial ice from the Antarctic with isotope concentrations of $-49‰$ $\delta^{18}O$ (SMOW) and $-400‰$ δD (SMOW). The other jar contained distilled ocean water with $+1.0‰$ $\delta^{18}O$ (SMOW) and $+17‰$ δD (SMOW). Both jars were placed in the chamber at the same time.

The experiment was repeated three times with new materials: once the growth chamber was maintained between 1.7 and 3.3 °C; once between 24 and 26.6 °C; and once the temperature fluctuated between 1.7 and 26.6 °C. Each time the oats in the jar containing water depleted in the heavy isotopes showed germination 1–2 weeks earlier and seemed to grow better throughout the growth period, than oats grown in distilled ocean water.

Using oats grown at 15 °C, the first sign of germination in the jar containing water depleted in the heavy isotopes was 4 d after planting. On the day 6, eight plants (out of 25) had attained a height of 6 cm. The first sign of germination in the jar with water containing the heavier isotope concentration, was after 17 d. By the time five plants had attained a height of 6 cm in this jar, in that with water depleted in the isotopes, 23 plants that had reached the top of the jar (approximately 25 cm).

Kashutin[1] observed that snow-water depleted in D increases the yield of cucumbers, radishes and spring wheat compared with controls grown in ordinary water of unspecified isotopic composition. He cites experiments on the egg productivity of hens and the weight gain of suckling pigs. In both cases water depleted in D was especially efficient in promoting productivity.

Although much has been done on the effect of D-enriched water on biological systems, we suggest that research on the effect D-depleted water on plant and animal growth may prove fruitful. A major source of water depleted in D by over 400‰ (40%) compared with SMOW is snow and ice from the Antarctic polar plateau. Water depleted by 150–180‰ is readily available in the USA from Rocky Mountain snow precipitating above 10,000 feet elevation.

JIM D. GLEASON
IRVING FRIEDMAN

US Geological Survey,
Denver, Colorado 80225

1 Kashutin, K, *Piiroda (USSR)*, 58, 107 (1969).

Identification of chlorinated dibenzofurans in American polychlorinated biphenyls

MORTALITY of embryos has contributed to the reproductive failures of several bird species, including the sparrowhawks (*Accipiter nisus*) of southern Scotland[1], the white-tailed eagles (*Haliaeetus albicilla*) of Schleswig Holstein[2], and the herring

gulls (*Larus argentatus*) of Lake Ontario[3]. Suspected causes include p,p'-DDE (2,2-*bis*-(p-chlorophenyl)-1,1-dichloroethylene), other chlorinated biocides and/or their derivatives, and the polychlorinated biphenyls (PCBs), all of which are present as contaminants in the eggs[1-3]. PCBs are present in high concentrations in the bird populations which suffer embryonic mortality[1-3]. Other organochlorine compounds which may be present in food webs include the chlorinated dibenzodioxins and the chlorinated dibenzofurans (Fig. 1), which are toxic to embryos in amounts[4-7] less than 1 μg. They are therefore among the most toxic substances known and are possible causes of the observed mortality.

The chlorinated dibenzodioxins and chlorinated dibenzofurans, however, have proved exceedingly difficult to detect in environmental samples in the concentrations at which they are expected to be embryotoxic[8-10]. The chlorinated dibenzodioxins enter the environment as contaminants in preparations of the herbicide 2,4,5-T (refs 5 and 11) and the fungicide pentachlorophenol[12,13]. Chlorinated dibenzofurans have been found in a French (Phenoclor DP6) and a German (Clophen A60) PCB and were shown to be the active embryotoxic agent in these preparations[6]. The techniques used, however, did not detect chlorinated dibenzofurans in an American PCB, Aroclor 1260. We report here the presence of chlorinated dibenzofurans in Aroclor PCB, widely used in North America and Great Britain, and in the same Aroclor 1260 preparation examined previously with negative findings[6].

Samples of PCB examined include: Aroclor 1248, 1254, and 1260 (1969); Aroclor 1254 (1970); Aroclor 1016 (1972); and the same three preparations studied by Vos et al.[6]: Aroclor 1260, lot No. AK-3; Clophen A-60, lot No. 912434; and Phenoclor DP-6, lot not specified. The latter three PCBs were obtained from Dr Vos, the others from the Monsanto Company in the years indicated in parentheses.

PCBs extracted from environmental samples most often have gas chromatographic profiles similar to those of PCB formulations containing approximately 48, 54 or 60% chlorine. In the Aroclor series, the former two PCBs are equivalent to Aroclor 1248 and Aroclor 1254, respectively. Aroclor 1260, Phenoclor DP6, and Clophen A60 all contain approximately 60% chlorine.

Chlorinated dibenzofurans were identified in all Aroclor preparations except Aroclor 1016, as well as in Clophen A60 and Phenoclor DP6. Aroclor 1016 is a PCB mixture containing
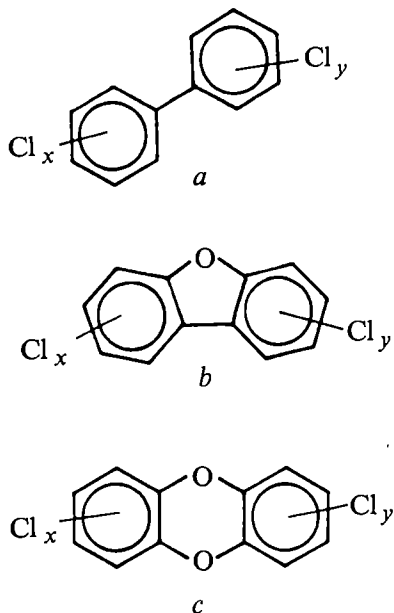
Table 1 Chlorinated dibenzofuran concentrations* in Aroclor, Clophen and Phenoclor

PCB	4-Cl	5-Cl	6-Cl	Total
Aroclor 1248 (1969)	0.5 (25)	1.2 (60)	0.3 (15)	2 0
Aroclor 1254 (1969)	0.1 (6)	0.2 (12)	1.4 (82)	1.7
Aroclor 1254 (1970)	0.2 (13)	0.4 (27)	0 9 (60)	1.5
Aroclor 1260 (1969)	0.1 (10)	0.4 (40)	0.5 (50)	1.0
Aroclor 1260 (lot. AK3)	0.2 (25)	0.3 (38)	0.3 (38)	0.8
Aroclor 1016 (1972)	ND	ND	ND	—
Clophen A-60	1.4 (17)	5.0 (59)	2.2 (26)	8.4
Phenoclor DP-6	0.7 (5)	10.0 (74)	2.9 (21)	13.6

*Expressed as μg g^{-1} PCB. Values in parentheses represent quantity as percentage total dibenzofuran.
ND, not detected (< 0.001 μg g^{-1}).

Amounts of PCB ranging from 1.0 to 2.0 g were dissolved in 400 ml hexane, placed on a Florisil column (180 g, internal diameter 31.5 mm), and eluted with: an additional 1,600 ml hexane, and successive 800 ml volumes each of 5% diethyl-ether–hexane, 25% diethyl-ether–hexane and acetone, at a rate of approximately 7 ml min^{-1}. The major portion of the PCB was eluted in the hexane fraction, which was discarded. On addition of the 5% mixture, the eluates were collected in six successive 400 ml volumes. To eliminate the polar solvents, each eluate was evaporated twice just to dryness and taken up each time in a minimal amount of hexane Each fraction, in 1 ml hexane, was placed on a microalumina column[14] and eluted with 10 ml each of 1% and 20% methylene chloride in hexane. These were also taken twice just to dryness and made to up a volume of 1 ml in hexane to eliminate the methylene chloride before gas chromatographic analysis. Aliquots of all fractions obtained before and after partitioning on alumina were injected into a six foot glass column containing 3% OV1 on 100–120 mesh Supelcoport in Tracor MT220 and Hewlett–Packard 5700 gas chromatographs equipped with ^{63}Ni electron-capture detectors. PCBs were found to be present in each fraction eluted from the Florisil column in amounts sufficient to interfere with the detection of trace contaminants. Partitioning on the alumina columns separated most of the PCB interference into the 1% methylene chloride fractions. On removal of this interference, different peak patterns appeared in the chromatograms of the 20% methylene chloride fractions. Compounds eluting in the 20% methylene chloride fraction were collected for mass spectrometric analysis using a 20:1 effluent splitter, and a trap consisting of a capillary tube (1 mm internal diameter, 100 mm long) bent to a U shape, immersed in a liquid nitrogen bath. Methylene chloride (20%; 4 μl) in hexane was injected into the capillary as a rinse, removed with a 1.0 μl micropipette, and placed directly on the mass spectrometer probe. The probe was inserted into a GEC AEI MS902 high resolution mass spectrometer and the solvent removed by the force pump. The probe was rapidly inserted into the ion source and multiple scans were recorded in the on-line high resolution mode[15].

Fig. 1 Skeletal structures of: *a*, chlorinated biphenyl, $x+y = 1$–10; *b*, chlorinated dibenzofurans, $x+y = 1$–8; *c*, chlorinated dibenzodioxins, $x+y = 1$–8.

approximately 42% chlorine and has replaced Aroclor 1242 in many applications, principally as the dielectric fluid in capacitors[16]. Values reported in Table 1 represent the total of those compounds found in 400 ml Florisil fractions 2–6. A total of 10–12 isomers was identified in each PCB. Two chlorinated dibenzofuran contaminants have been reported for the Clophen and Phenoclor previously[6]; our first analyses of the Clophen revealed an additional five chlorinated dibenzofurans[8]. The structures contained four to six chlorine atoms. Other dibenzofurans including those chlorinated to a lesser extent may have been present in the first 400 ml fraction but this was not examined in detail as it contained substantial PCB interference. Recently synthesised 2,3,7,8-tetra-, 2,3,4,7,8-penta- and 2,3,4,6,7,8-hexachlorodibenzofuran were used to quantify tetra-, penta-, and hexachlorodibenzofurans, respectively. The former two authentic standards had retention times on the OV1 column the same as those of two dibenzofurans isolated from the PCB.

Vos et al.[6] detected no chlorinated dibenzofurans in an Aroclor 1260 preparation at a detection limit of 1 p.p.m. Fractionation and examination of the identical Aroclor 1260 in our study confirm their findings based on the stated limit, but reveal the presence of 11 chlorinated dibenzofurans in the preparation, having a total concentration of 0.8 μg g^{-1} PCB (Table 1). The same workers also found diethyl ether extracts of the Clophen A60 and Phenoclor DP6 to be much more toxic to chick embryos than diethyl ether extracts of Aroclor 1260. Our study confirms those findings on the basis of chlorinated
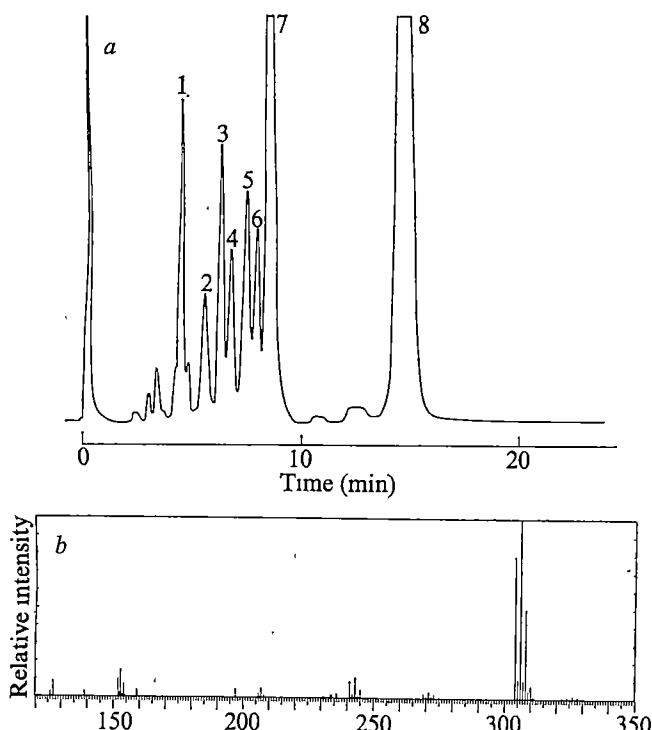
Fig. 2 *a*, Gas chromatogram of a fraction of Aroclor 1254 containing a mixture of chlorinated biphenyls, dibenzofurans, and naphthalenes Identities of peaks are given in the text. *b*, Mass spectrum of peak 2, a tetrachlorodibenzofuran.

dibenzofuran content: the identical Clophen and Phenoclor contain 11 and 17 times more total chlorinated dibenzofurans, respectively, than the Aroclor 1260.

A gas chromatogram showing components derived from the Aroclor 1254 obtained in 1969 is represented in Fig. 2. The components were eluted in the second 400 ml Florisil fraction and recovered from the alumina column in 20% methylene chloride–hexane. Each of the numbered peaks was trapped as described here, and identified by mass spectrometric analysis. A nominal mass plot of the high resolution mass spectrum of peak 2 is shown in Fig. 2. The plot includes all the ions with elemental compositions ranging to the maximum empirical formula $C_{13}H_6O^{35}Cl_5^{37}Cl_4^{13}C_2$. The molecular ion cluster at nominal m/e 304–310 fragments by successive losses of Cl to yield the ions at m/e 269–275 and CO to the ions at m/e 241–245. A minor loss of Cl from the peaks at m/e 269–275 also occurs to yield the ions at m/e 234–238, followed by CO elimination to m/e 206–210.

The group of peaks at m/e 152–154 are the doubly charged molecular ions. An identical spectrum was obtained from an authentic standard of 2,3,7,8-tetrachlorodibenzofuran. This latter compound has a retention time identical to that of peak 4. Peak 2 is, therefore, a positional isomer. The accurate mass measurements for the characteristic ions are within 2 p.p.m. of the calculated exact masses. Peaks identified on this chromatogram and their retention times relative to dieldrin are as follows: a mixture of tetra- and pentachlorobiphenyl (1.02); tetrachlorodibenzofuran (1.30); pentachlorobiphenyl (1.46); tetrachlorodibenzofuran (1.57); hexachloronaphthalene (1.75); pentachlorobiphenyl (1.86); hexachloronaphthalene (2.00); and heptachloronaphthalene (3.46). An aliquot of combined fractions derived from the same Aroclor 1254 was treated with diazomethane to assess whether any chlorinated ortho-hydroxy-biphenyls (pre-furans) were present. Gas chromatographic analysis of the sample before and after treatment resulted in identical chromatograms.

As large quantities of PCBs have entered the global environment[17-20], it may be assumed that the contaminant dibenzofurans

also have been released in proportional amounts. Their persistence, effects, and significance remain to be determined.

We thank J. A. Burke, M. L. Porter and J. G. Vos for discussions; A. S. Kende for standards of chlorinated dibenzofurans; and F. C. Walls for assistance with the mass spectrometry. This work was supported by the Canadian Wildlife Service, National Science Foundation, and NASA.

GERALD W. BOWES*
MICHAEL J. MULVIHILL

Canadian Wildlife Service,
Toxic Chemicals Section,
Ottawa, Canada K1A OH3

BERND R. T. SIMONEIT
A. L. BURLINGAME

Space Sciences Laboratory,
University of California,
Berkeley, California 94720

R. W. RISEBROUGH

Bodega Marine Laboratory,
University of California,
Bodega Bay, California 94923

1 Newton, I , and Bogan, J., *Nature*, 249, 582–583 (1974).
2 Koeman, J. H , Hadderingh, R. H., and Bijleveld, M. F. I. J., *Biol. Conserv.*, 4, 373–377 (1972).
3 Gilbertson, M., and Hale, R., *Can. Fld Nat.*, 88, 354–356 (1974).
4 Higginbotham, G. R., *et al.*, *Nature*, 220, 702–703 (1968)
5 Sparschu, G. L., Dunn, F. L., and Rowe, V. K., *Food Cosmet. Toxic.*, 9, 405–412 (1971).
6 Vos, J. G , Koeman, J. H., Van der Maas, H. L., ten Noever de Brauw, M. C., and de Vos, R H , *Food Cosmet. Toxic.*, 8, 625–633 (1970).
7 Vos, J G , *Environ Hlth Persp.*, 1, 105–117 (1972).
8 Bowes, G. W , Simoneit, B. R., Burlingame, A. L , de Lappe, B. W., and Risebrough, R. W , *Environ. Hlth Persp.*, 5, 191–198 (1973).
9 Baughman, R. and Meselson, M., *Environ Hlth Persp.*, 5, 27–35 (1973).
10 Baughman, R., and Meselson, M., *Adv. Chem.*, 120, 92–104 (1973).
11 Report on 2,4,5-T (Executive Office of the President, Science Advisory Committee, Office of Science and Technology, March, 1971)
12 Jensen, S., and Renberg, L., *Ambio*, 1, 62–65 (1972)
13 Firestone, D., Ress, J., Brown, N. L., Barron. R. P , and Damico, J. N., *J. Ass. Off Analyt. Chem.*, 55, 85–92 (1972).
14 Porter, M L., and Burke, J. A., *J Ass. Off Analyt. Chem.*, 54, 1426–1428 (1971).
15 Burlingame, A L , Olsen, R. W. and McPherron, R. V., *Adv. Mass Spectr*, 6, 1053–1059 (1974)
16 Nisbet, I C. T., and Sarofim, A. F., *Environ Hlth Persp.*, 1, 21–38 (1972).
17 Bowes, G. W , and Jonkel. C J , *J. Fish Res Bd Can* (in the press)
18 Jensen, S , Johnels, A. G., Olsson, M., and Otterlind, G., *Nature*, 224, 247–250 (1969).
19 Koeman, J. H , ten Noever de Brauw, M. C., and de Vos, R. H., *Nature*, 221, 1126–1128 (1969).
20 Risebrough, R W., Reiche, P., Peakall, D. B , Herman, S. G , and Kirven, M N , *Nature*, 220, 1098–1102 (1968).

Niche breadth in Bryozoa as a test of competition theory

COMPETITION theory predicts that intraspecific and interspecific competition should often have opposite effects on the use of resources by a population, the former increasing, the latter decreasing, the range of resource actually used[1,2]. Field data supporting these predictions are well known for the interspecific case[3,4] but are scarce for the intraspecific condition, and we have been unable to find any reference demonstrating both effects within a single species. We therefore report here the verification of both predictions in respect of competition for space by the epiphytic bryozoan *Alcyonidium hirsutum;* less extensive data suggesting the same effects within other bryozoans are also reported.

Intraspecific competition should result in an increase in the range of a resource spectrum used by a species, as 'at high population levels the advantages to any individual of being at the competition-free optimum of a resource gradient are offset by the intense intraspecific competition found there (Fig. 1a); this is the 'principle of equal opportunity' of MacArthur[1]. Interspecific competition, on the other hand, should tend to restrict the range of the resource spectrum used by a species, as individuals attempting to exploit marginal resources cannot do so as efficiently as

individuals of other species for which these resources are more nearly optimal; niche width on the resource gradient is thus reduced. Furthermore, such reduction can be expected to increase with increase in the number of competing species, as a greater proportion of the original resources is then nearer the optimum for a competing species (Fig. 1b); this is the diffuse competition situation of MacArthur[1,2]. These predictions will have been confirmed if it can be shown that the range of a resource spectrum used by a species increases with population density but decreases as the number of competing species increases.

Our analysis is based on the distributions of Bryozoa on plants of the serrated wrack *Fucus serratus*. Sixty-five plants were collected in April, 1972 near Ardkeen, County Down, Northern Ireland, and the longest frond of each divided into Y-shaped dichotomies, labelled Y1, Y2, and so on, from holdfast to tip. The percentage cover by each bryozoan species was recorded for each of the two faces of each dichotomy. The commonest bryozoans were *Alcyonidium hirsutum, Electra pilosa, Flustrellidra hispida* and *Membranipora membranacea;* other bryozoan species were sufficiently rare to be ignored in this analysis. Fuller details of our methods have been recorded elsewhere[5].

Fig. 1 Niche breadth changes under the influence of intraspecific (a) and interspecific (b) competition. In a, high density (HD) curve is broader than is the low density (LD) curve as more individuals settle at more marginal but less hotly contested points on the resource gradient. In b, presence of competitors utilising resources on both sides of a species' optimum on the resource gradient compresses that species' niche breadth.
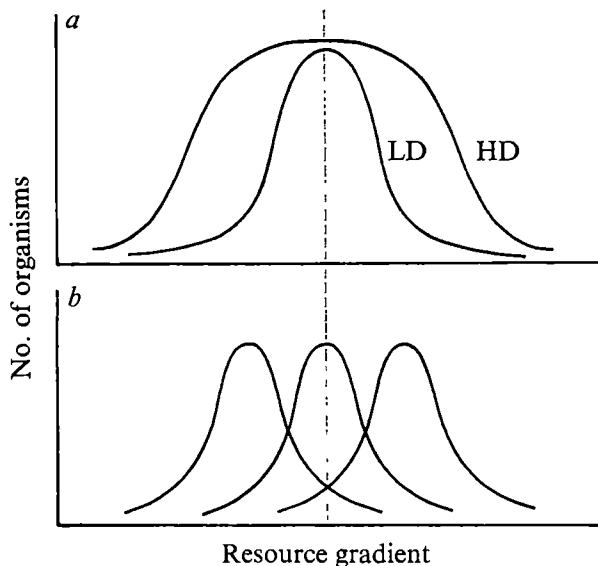
In an epiphytic community of filter-feeders such as that found on *F. serratus*, competition for space is of major significance: each fucoid frond constitutes a linear resource which can be partitioned longitudinally among the competing species[5,6]. We therefore tested MacArthur's principle of equal opportunity by plotting the longitudinal range of *Alcyonidium* on each plant against its population density on that plant, using only the fourteen plants on which it was the sole bryozoan present and thus eliminating the effects of interspecific competition from other bryozoans. The results (Fig. 2) show that *Alcyonidium* used a greater range of Y values (height when the plants are upright, as an index of distance from the holdfast) at high population densities than at low densities. As the available space at the optimum height was not physically filled as populations increased this must result from competitive interactions between colonies[7], thus confirming the prediction of increased range of resource use. Note that
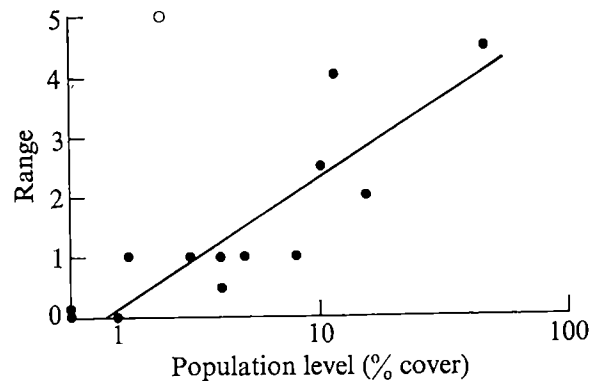
Fig. 2 Niche breadth in relation to population level for *Alcyonidium* in the absence of competition from other bryozoa. Niche breadth estimated as the range in Y value of colonised segments, calculated separately for each side of a frond and averaged. Population level is estimated as the mean percentage cover of all colonised segment faces of the plant. O, Anomalous result discussed in text. Regression is significant ($r = 0.631, P < 0.05$).

one plant gave anomalous results because of extremely distal settlement of a very few zooids (Fig. 2); the regression is statistically significant when this point is included ($r=0.631$, $P<0.05$) but on omitting it the correlation rises to 0.890 ($P<0.01$), thus almost doubling the proportion of variance in resource span accounted for by population density.

Too few plants were colonised by another single bryozoan species to allow precisely the same analysis as for *Alcyonidium*. For these other species we therefore included plants on which any one other bryozoan was present even though this introduced some interspecific competition tending to reduce niche breadths (see below) and thus to mask the intraspecific effect. Nevertheless, for each species a positive correlation between range and density per plant (on a log scale) was obtained (*Electra, r*=0.595, *P*<0.025; *Flustrellidra, r*=0.363, *P*<0.30; *Membranipora, r*=0.606, *P*<0.09), thus suggesting a niche widening effect for intraspecific competition in these species also.

It is in principle possible for the results above to have arisen through a pattern of age-dependent settlement by Bryozoa on growing *Fucus* fronds[5]. We therefore examined

Fig. 3 Resource utilisation curves for *Alcyonidium* with different numbers of competing Bryozoa present. Ordinate, number of *Fucus* segment faces colonised; abscissa, Y value. ●—●, Faces colonised by *Alcyonidium* alone; □—□, faces colonised by *Alcyonidium* and any one other bryozoan species; ●...●, faces colonised by *Alcyonidium* and two other bryozoans.
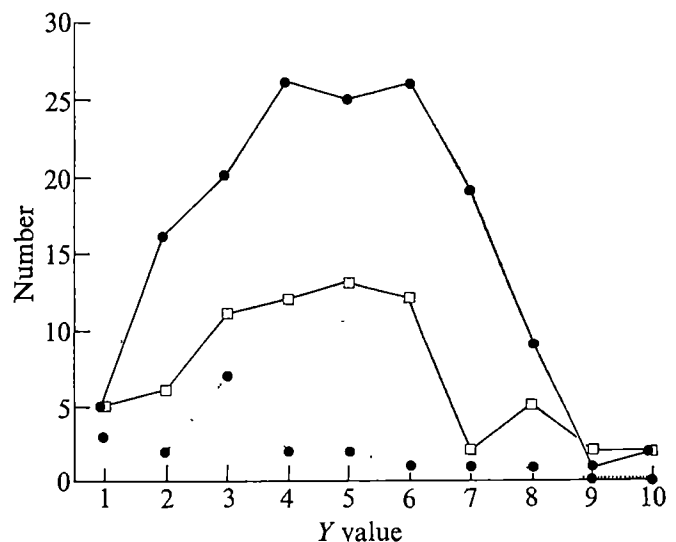
Table 1 Effect of interspecific competition on niche breadth in Bryozoa

Number of competing species present	Alcyonidium		Electra		Flustrellidra		Membranipora	
	Breadth	Cases	Breadth	Cases	Breadth	Cases	Breadth	Cases
0	5.9	149	5.1	59	6.8	51	5.0	41
1	4.4	69	2 6	58	6.1	57	1.7	18
2	2.7	18	1.6	20	1.6	20	1.0	2

Niche breadth was assessed from plots such as those of Fig. 3 as the width of the resource utilisation function at half-height. Cases column shows the sample size on which each function was based. The second and third rows are based on all cases satisfying the row criterion, irrespective of the identity of the competitor(s).

the distribution of each species on each plant in the light of the age of that plant as indexed by the number of dichotomies on the longest frond: no systematic pattern was apparent in any case, thus precluding that explanation.

The effects of interspecific competition on *Alcyonidium* niche breadth were examined by plotting the distribution of segment faces colonised by *Alcyonidium* with (in turn) no other, one other and two other bryozoan species on the same face. The results (Fig. 3) show that *Alcyonidium* used a wider range of heights on the plants in the absence of competition than in its presence, and used a wider range when competing with one species at a time than with two. These results are summarised with those for the other three species in Table 1, using the width at half-height of each resource utilisation function as an index of niche breadth; the standard deviations used for this purpose by MacArthur[1] were not appropriate with the non-Gaussian distribution found here[8]. Table 1 shows that niche breadth was reduced progressively in all four species as the number of competing species increased, thus confirming the theoretical prediction for the effects of interspecific competition. Note that these results did not arise as artefacts of partial overlapping of largely segregated resource utilisation functions[5].

A number of previous studies, particularly those demonstrating ecological release in island populations of birds[3,4], have shown interspecific competition to restrict niche breadth. The effect of intraspecific competition in increasing niche breadth has been less well documented, although it can be inferred from the 'buffer effect' of population studies, in which marginal habitats are utilised by territorial animals only at high population levels[9]. These two types of study have rarely been combined to contrast the effects of interspecific and intraspecific competition within a single species, probably because of the practical difficulties of assessing population density and resource use simultaneously. The more rigorous test of competition theory reported here was possible because of the special features of the group studied, bryozoans on *Fucus* competing along what is effectively a single resource dimension on which their densities are readily assessed. A detailed account of habitat partitioning in bryozoans will be published elsewhere.

R. J. O'CONNOR
P. J. S. BOADEN
R. SEED

*Department of Zoology and Marine Biology Station,
The Queen's University of Belfast,
Belfast BT7 1NN, Northern Ireland*

1 MacArthur, R H., *Geographical Ecology* (Harper and Row, New York, 1972).
2 Pianka, E R , *Evolutionary Ecology* (Harper and Row, New York, 1974).
3 Crowell, K. L. *Ecology*, 43, 75–88 (1962).
4 Lack, D , *Ibis*, 86, 260–286 (1944)
5 Boaden, P. J S , O'Connor, R J., and Seed, R., *J exp. mar. Biol. Ecol.*, 17, 111–136 (1975)
6 Boaden, P. J. S , O'Connor, R J., and Seed, R., *J exp. mar. Biol Ecol.* (in the press)
7 Ryland, J. S., *Bryozoans* (Hutchinson, London, 1970).
8 Cody, M. L , *Competition and the Structure of Bird Communities* (Princeton University Press, Princeton, 1974).
9 Krebs, J. R., *Ecology*, 52, 2–22 (1971)

Hue is an absolute code for young children

THE question of how young children see, remember, and learn represents a recurring theme in psychology. Bryant[1] has argued provocatively that "young children can on the whole register and remember relative values with great ease, but have problems in situations in which they must remember absolute values along any continuum". Bryant shows[1] how this view of early cognitive functioning accounts for the results from studies of children's responses to several environmental continua of a perceptual nature (for example, orientation, size, and position). I have, however, observed that young infants respond to changes of wavelength in a manner which indicates that they code hue absolutely.

Bryant has reanalysed extant studies and described new data which show that children perform well at simultaneous discrimination tasks (where comparison stimuli are available at the same time as a standard) whereas they perform poorly at successive discrimination tasks (where choice stimuli are available after a standard has been withdrawn). On the basis of this evidence, Bryant proposed that children use relative codes to make simultaneous comparisons. Successful performance in successive comparisons requires a constant external framework or an absolute code of stimulus characteristics; in general, Bryant has found that where mediating environmental features were wanting children's performances at successive discriminations fell to chance. Relative codes rather than absolute codes, he concluded, are therefore primary in young children, and the growing reliance on absolute codes, if it occurs at all, is a late developmental phenomenon. In this view, man's initial perception and memory of environmental stimuli are dominated and controlled exclusively by their relationships to each other or to surrounding frames of reference.

Contrary to Bryant's analysis, for a stimulus dimension (wavelength) which conforms to his investigative requirements (both absolute values of stimuli and relationships among stimuli can be specified) and in an experimental situation (successive discrimination) which fits his physical requirements (luminous figures were presented without a frame of reference in an otherwise dark room), infants in my studies were observed to use absolute codes in successive discrimination. That is, infants responded to hues on an absolute basis with no alternatives other than those which may be inferred to be internal categories of information. This is much the way that adults respond to hue[2-3].

Ten infants (mean age, 124 d) were first familiarised with a 480 nm stimulus: they were shown a light—which appears to the adult mostly 'blue' with some 'green'— serially for 15 trials each of 15 s duration. During this

familiarisation phase (and during the test phase which followed) the infants' observation of the light was monitored by observers unaware of the stimulus wavelength (judgment reliabilities: 0.93< r <0.97), and the total observation times per trial were later scored blindly from event records. Typically, familiarisation to one stimulus leads to shorter periods of observation on successive presentations of that stimulus; after familiarisation, continued exposure to the original stimulus or to a different stimulus coded as the same as the first leads to a continued depressed level of looking, whereas exposure to a different stimulus coded differently from the first leads to increased visual attention[4]. Immediately following the familiarisation phase, infants experienced nine test trials (each of 15 s duration) in which they saw 450 nm, 480 nm, and 510 nm stimuli serially in three different random orders. Thus, in the test phase, infants saw the familiarisation stimulus (480 nm or 'greenish-blue') and two new stimuli: 450 nm or 'blue' and 510 nm or 'green'. Adults see and code 450 nm and 480 nm as similar and as drawn from the same basic hue category, but they see and code 480 nm and 510 nm as different and as drawn from different basic hue categories. This experiment (Table 1) showed that infants looked at 450 nm and 480 nm for an equal and relatively little amount of time but that they looked at 510 nm for significantly longer ($F=16.77$, $P<0.001$, by a planned comparisons analysis[5]).

In a successive discrimination task in a situation lacking external frames of reference, infants responded to equal extents to a wavelength with which they had been previously familiarised and to another from the same basic hue category, which they had not seen previously; but they responded to a greater extent than that to a wavelength which they had not seen previously and which was drawn from a different hue category. Internal controls showed that differential responses could not be a function of fluctuation in attention (the familiarisation stimulus appeared randomly during the test) or simple change in wavelength (the two new stimuli were equally distant in wavelength from the familiarisation stimulus). Moreover, in a similar successive discrimination task, infants who were familiarised with a luminous oblique line did not discriminate it from its mirror image; that is, they gave equal amounts of attention to both. It is clear from this pattern of results that infants can code wavelength absolutely by apparent hue and can respond to changes in wavelength with respect to a set of basic absolute hue codes, whereas they cannot code orientation absolutely[1].

It is an explicit claim of Bryant's theory that young children, possessing exclusively relative codes, should be able to perform successive discrimination tasks and concomitant perceptual inferences only in the presence of a constant frame of reference. Without such a reference, absolute codes are necessary, and lacking such codes children perform at chance. The possession by infants of an absolute code for hue (and for certain speech sounds[6]) represents, however, a case which Bryant happily provided for: "Abilities which have been definitively ruled out in young children by one psychologist have a habit of cropping up in the experiments of another". The presence of hue codes in young infants helps us further understand the

nature of early sensory and perceptual processes and of the demonstrative predominance of colour over form in young children's natural classification systems[7].

MARC H. BORNSTEIN

Department of Psychology,
Yale University,
New Haven, Connecticut 06520

1 Bryant, P. E., *Perception and Understanding in Young Children* (Basic Books, New York, 1974).
2 Bornstein, M. H., *Psychol.Bull.*, 80, 257 (1973).
3 Boynton, R. M , *Woodworth and Schlosberg's Experimental Psychology* (edit. by Kling, J , and Riggs, L. A.), 315 (Holt, Rinehart, and Winston, New York, 1973).
4 Jeffrey, W. E , and Cohen, L B., *Adv Child Dev. Behav.*, 6, 63 (1971).
5 Winer, B. J., *Statistical Principles in Experimental Design*, 384 (McGraw-Hill, New York, 1971)
6 Eimas, P. D., Siqueland, E., Jusczyk, P , and Vigorito, J., *Science*, 171, 303 (1971).
7 Suchman, R. G., and Trabasso, T , *J. exp. Child Psychol.*, 3, 177 (1966).

Sexual transfer of specific genes without gametic fusion

THE most common method of combining genes from two organisms is by sexual hybridisation. In plant breeding, however, a variety can often be improved considerably by introducing only one or a small number of specific genes. Elimination of accompanying undesirable characteristics normally requires several generations of backcrossing and selection which may greatly prolong the breeding period. Here I describe experimental results which suggest a possible new technique for more rapid transfer of desired genes between two organisms.

During experiments on the role of 'mentor pollen' in overcoming intraspecific incompatibility in *Nicotiana*, some unexpected observations were made. In some cases, as expected the use of 'killed' irradiated (100,000 rad, Co : 347.6 rad min^{-1}) mentor pollen mixed with the viable maternal incompatible pollen led to the breakdown in the self incompatibility system resulting in the production of selfed progeny. (Mentor pollen is compatible irradiated pollen which produces a growth tube but fails to fertilise the ovule. In certain genotypic combinations substances are released by the mentor pollen which allow normally incompatible pollen to produce a growth tube able to fertilise the ovule.) In other cases the mentor pollen had no effect and usually no seed was produced. But in these latter combinations, which involved the related but morphologically distinct intercompatible species *N. forgetiana* and *N. alata*, in a few cases rare viable seeds were produced along with a large number of collapsed non-viable ovules.

These seeds gave rise to 24 normal, fertile, diploid plants which, in general morphological characteristics, all resembled their maternal parents. Surprisingly, however, most of these plants showed coloured flowers and/or an incompatibility allele characteristic of the mentor pollen source (Table 1). Fourteen of the 24 plants were examined cytologically and all were found to be normal. No additional chromosome fragments[1-3] were present.

To investigate this apparent 'gene transfer' phenomenon, 14 plants showing flower colour and/or an S allele typical of the mentor pollen source were crossed with various tester plants of known genotypes (Tables 2 and 3). The most significant result of these test crosses was the recovery of 34 plants carrying 3 S alleles from crosses C2-1, C2-2, C2-3, D6-1 and D7-1 (Table 3). Triallelic plants of this type cannot be obtained by any normal form of inheritance and their appearance is strong evidence for the occurrence of an unusual genetic transfer process

A number of other anomalous features of these experimental results also suggest the transfer of genes from irradiated mentor pollen without normal gamete fusion :
(1) Although the exceptional plants may have been derived from rare pollen nuclei which survived the irradiation

Table 1 Mean looking times(s)

Test wavelengths* (nm)	Mean	S.d.
450	5.8	2.7
480	5.7	2.8
510	7.3	2.4

*Luminances = 3.4 cd m^{-2}.

Table 1 Initial observations suggesting gene transfer in experiments involving the use of irradiated mentor pollen to overcome self incompatibility

Family code	Cross — Selfed female parent *S* genotype and flower colour	Cross — Mentor pollen source *S* genotype and flower colour	Total no. of plants*	Progeny‡ No. of plants with stated *S* genotype and flower colour (italics denote 'transferred' character)			Cytology†
A	*N. forgetiana* $S_{F1}\,S_{F2}$ White (rr)	*N. alata* $S_3\,S_3$ Red (Rr)	7 (A1 to A7)	4	$S_{F1}\,S_{F1}$	*Red*	Normal (3 plants)
				2	$S_{F2}\,S_{F2}$	*Red*	
				1	?	*Red*	
B	as above		4 (B1 to B4)	1	$S_{F1}\,S_{F1}$	*Red*	Normal (3 plants)
				1	$S_{F2}\,S_{F2}$	*Red*	
				2	$S_{F2}\,S_{F2}$	White	
C	*N. alata* $S_{F10}\,S_{F11}$ White (pp)	*N. alata* $S_1\,S_3$ Pink (Pp)	5 (C1 to C5)	1	$S_1\,S_{F10}$	*Pink*	Normal (3 plants)
				1	$S_1\,S_{F11}$	*Pink*	
				1	$S_3\,S_{F11}$	*Pink*	
				1	$S_3\,S_{F11}$	White	
				1	S? S_{F11}	White	
D	as above		8 (D1 to D8)	1	$S_3\,S_{F11}$	*Pink*	Normal (5 plants)
				2	$S_3\,S_{F11}$	White	
				1	$S_3\,S_{F10}$	White	
				3	$S_1\,S_{F10}$	White	
				1	$S_1\,S_{F11}$	White	

*Families were derived from single pods except for (B) which was derived from two pods with two seeds in each. Normally a pod contains up to 1,000 or more seeds.

†Only 14 of the 24 plants were examined cytologically.

‡To overcome the problem of breakdown of incompatibility reaction in parthenogenetic diploids when tested with highly heterozygous testers[5], only appropriately inbred plants were used as *S* gene testers in these experiments.

treatment, the extremely high lethal dose of 100,000 rad has a margin of error which virtually rules out this possibility. In addition, any plant arising from fertilisation by an irradiated nucleus would be expected to show extreme sterility, lack of vigour, or gross morphological defects. No such effects were observed.

(2) In families A and B (Table 1) where the mentor pollen was obtained from a different species, the progeny were unequivocally maternal in floral and general morphology, and were definitely distinct from normal F_1 *N. forgetiana* × *N. alata* hybrids.

(3) In the same families, A and B, in which the mentor pollen parent was homozygous for the S_3 gene, the progeny were all homozygous for one or other maternal *S* allele, whereas if they arose through normal fertilisation by mentor pollen they would all have carried the S_3 allele, And yet the participation of the mentor pollen parent is unequivocal, for the red flower colour of the progeny could have come only from that parent.

(4) Also in families A and B, nine out of the 11 progeny were red-flowered, whereas according to the genotypes of the parents a true hybridisation should have given a ratio of 1 red to 1 white.

(5) Contamination with foreign pollen cannot explain the anomalous plants in Table 1. First, the plants were all grown in an insect-proof greenhouse and experimental procedures were strictly controlled. Second, contamination would be expected to produce extremely rare pods with a full set of viable seeds, but in these crosses the pods were too frequent and the number of viable seeds per pod too low to represent accidental contamination.

(6) Although the anomalous plants from crosses A to D are highly fertile (60–9$\tilde{?}$% stainable pollen), some nevertheless show a greater degree of pollen sterility than is usually observed in normal cross-pollinated plants of this genus.

(7) Finally, a unique phenotypic abnormality was recovered among the progeny of four anomalous plants, C1, C2, C4 and D4. In these plants, which are otherwise normal in appearance, the 'floral' shoot became vegetative with tufts of small leaves in place of flower buds. When these plants were cut back to the stem base they regenerated the original

type of vegetative inflorescence. No such plants have previously been seen or reported in *Nicotiana*[4] to the author's knowledge. This novel phenotype was apparently generated as a consequence of unorthodox genetic recombination processes.

Table 2 Segregation among the progeny of plants from crosses A and B which apparently carried transferred flower colour genes

Family code	Cross* (italics denote transferred character) ♀	♂	Red	Segregation White	Expected† ratio
A2–1	A2 *Red* ×	white	17	15	1:1
2	[A2 *Red* ×	white]	5	3	1:1
3	[white ×	A2 *Red*]	21	9	1:1
A6–1	A6 *Red* ×	white	7	7	1:1
2	[A6 *Red* ×	white]	11	7	1:1
3	[white ×	A6 *Red*]	13	8	1:1
4	A6 *Red*	selfed	11	2	3:1
A7–1	A7 *Red* ×	white	7	6	1:1
2	A7 *Red* ×	white	3	7	1:1
3	[A7 *Red* ×	white]	13	15	1:1
4	[white ×	A7 *Red*]	9	5	1:1
B1–1	white ×	B1 *Red*	18	15	1:1
B4–1	B4 *Red* ×	white	24	16	1:1
2	[B4 *Red* ×	white]	19	17	1:1
3	[white ×	B4 *Red*]	19	7	1:1
4	Red (Rr) ×	B4 *Red*	31‡	4	3:1
5	B4 *Red*	selfed	29	4	3:1

*Testers were normal diploid plants, homozygous recessive for flower colour (rr). In B4-4 a heterozygous red-flowered tester (Rr) was used. Brackets indicate reciprocal crosses.

†Segregation expected if the transferred gene is heterozygous and behaves normally. 1:1 segregations. Crosses A2-3 and B4-3 show significant excesses of red-flowered progeny ($P < 0.05$). Overall there is a highly significant excess of red-flowered progeny ($P < 0.01$). 3:1 segregations. Individual segregations are not significantly different from expectation, but the overall data show a highly significant excess of red-flowered progeny ($P < 0.01$).

‡Includes normal and 'transferred' red flowered plants.

The following sequence of events is proposed as a working hypothesis to explain the above results.

The extremely high dose of irradiation given to the mentor pollen 'pulverises' the generative nucleus to produce a mass of fine chromatin fragments. Division of the generative nucleus fails, and this may contribute further to chromatin fragmentation. Discharge of the pulverised chromatin from the pollen tube into the egg acts as a 'pseudo-fertilisation'. The block to cell division in the egg is lifted as usual and the chromosomes are replicated, but the presence of disorganised pollen chromatin prevents a normal first zygotic division. This results in a diploid egg with the 'induced' physiology of a zygote. Presumably, at least in certain cells, the free chromatin fragments are rapidly degraded, or lost, and their disorganising effects are soon overcome to enable subsequent mitotic divisions to occur normally. The resulting embryo will be a parthenogenetic diploid. Rare occurrence of parthenogenetic diploidy after pollination with irradiated pollen has previously been reported in *Nicotiana*[5]. The slightly greater pollen sterility shown by some of the anomalous plants is mainly attributed to parthenogenetic diploidy exposing deleterious recessive genes.

Fragments of mentor pollen chromatin may occasionally associate with their homologues among the egg chromatin. These fragments will have broken 'sticky' ends and may insert into nicks formed during chromosome replication[6]. The following possibility is suggested to explain why the pollen chromatin fragments should be attracted to the homologous segments in the egg chromatin in a manner reminiscent of meiotic pairing. Once replication has been induced, the homologous pollen chromatin molecules are

able to compete successfully with the *de novo* synthesised molecules, since they occupy the respective positions next to the strand before the appropriate molecules could be freshly synthesised.

Once chromatin segments from pollen have paired with those of the egg, substitution or addition may easily occur during replication. Three types of chromatin transfer can be imagined (Fig. 1a, b, c). It is interesting to note that results consistent with these patterns of transfer have actually been found (Table 3) Plant D6 illustrates the triallelic state (Fig 1b), and plant C2 probably illustrates the alternative triallelic state (Fig. 1c). Plants C2 and D7—the third plant showing the triallelic state—illustrates the instability of the exosome, producing three types of gametes (Fig 1e). The remainder of tested plants in Table 3 represent the substituted state (Fig. 1a).

The gene transfers observed in this study seem to be fairly stable. No variegated or blotchy flower coloration, characteristic of *Petunia* transformations[7], was obtained. Only in certain rare cases are there noteworthy deviations from expected segregation ratios (Table 3). As regards flower colour, however, there may be a general tendency to recover an excess of coloured flowered plants bearing the transferred gene. This apparent selection in favour of the transferred gene is not related to the direction of the cross, thus ruling out any advantage in terms of pollen tube growth.

Previous 'transformation' studies[7-10] with higher organisms have so far been unable to distinguish unequivocally between substitution and addition models of chromatin transfer. In the present results, since the presence or absence of individual *S* alleles in the style can be determined
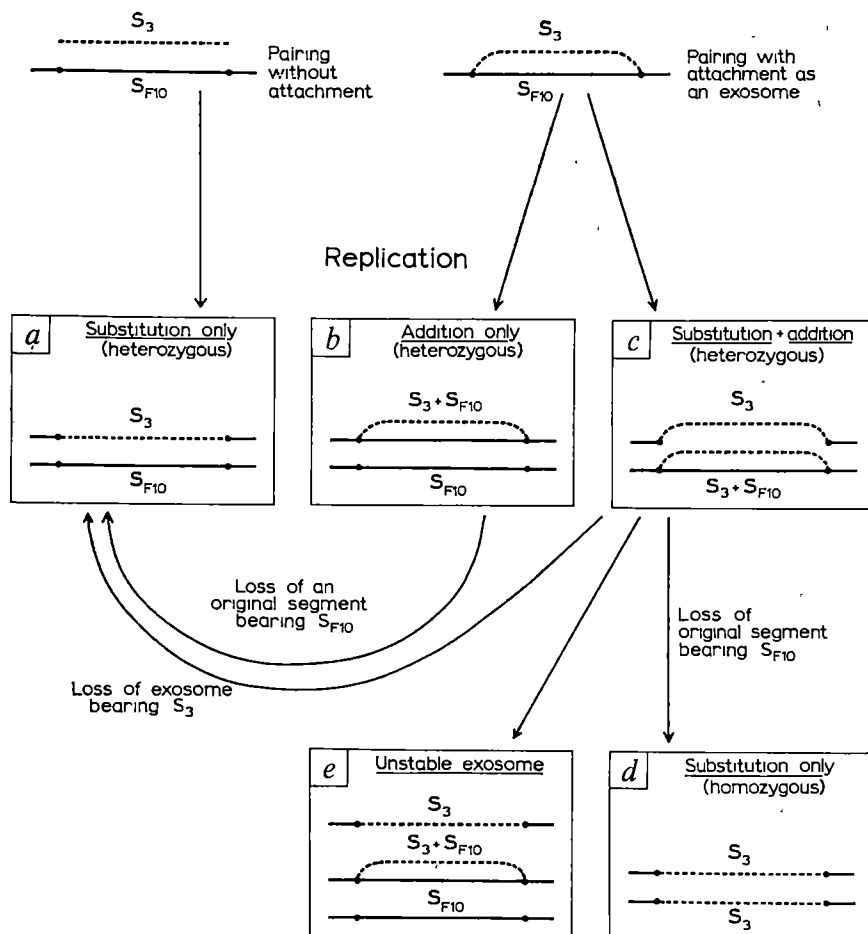
Fig. 1 Diagrammatic illustration of proposed patterns of gene transfer. Genetic states corresponding to *a*, *b*, *c* and *e* have been observed experimentally (see text). —————— Egg chromatin strand (or replicated copy). ------------- Irradiated pollen chromatin (or replicated copy). Single lines represent strands of 'chromatin', not single strands of DNA.

Table 3 Segregation among the progeny of plants from crosses C and D which apparently carried transferred flower colour and/or S-genes

Family code	Cross* (italics denote transferred character) ♀		♂		Normal	S genotypes Triallelic	Expected‡	Flower colour Pink	White	Expected‡
C1 —1	C1(S_3S_{F11}, *Pink*)	×	S_1S_1,Pp	10 (S_1S_3)§§	21 (S_1S_{F11})		1:1	25§	12	3:1
2	[C1	×	$S_{F10}S_{F11}$,pp]	13 (S_3S_{F10})	12 ($S_{F10}S_{F11}$)		1:1	16	11	1:1
3	[$S_{F10}S_{F11}$,pp	×	C1]	13 (S_3S_{F10})	9 (S_3S_{F11})		1:1	16	14	1:1
C2¶ —1	[C2(S_1S_{F11}, *Pink*)	×	$S_{F10}S_{F11}$, pp]	13 (S_1S_{F10})	10 ($S_{F10}S_{F11}$)	5 ($S_1S_{F10}S_{F11}$)		16	14	1:1
2	[$S_{F10}S_{F11}$ pp	×	C2]	9 (S_1S_{F10})	13 (S_1S_{F11})	9 ($S_1S_{F10}S_{F11}$)		24	8††	1:1
3	C2	×	S_3S_3Pp	19 (S_3S_{F11})		8 ($S_1S_3S_{F11}$)		15§	15‡‡	3:1
4	C2		selfed	4 (S_1S_1),	17 (S_1S_{F11}),	3 ($S_{F11}S_{F11}$)		23	3	3:1
C4† —1	C4(S_3S_{F11})	×S_1S_1		8 (S_1S_3)	6 (S_1S_{F11})		1:1			
2	$S_{F10}S_{F11}$	×	C4	4 (S_3S_{F10})	2 (S_3S_{F11})		1:1			
D1 —1	D1(S_3S_{F11}, *Pink*)	×$S_{F10}S_{F10}$,pp		9 (S_3S_{F10})	10 ($S_{F10}S_{F11}$)		1:1	11	8	1:1
2	D1	×	S_1S_1,Pp	15 (S_1S_3)	13 (S_1S_{F11})		1:1	25§	9	3:1
3	$S_{F10}S_{F11}$,pp	×	D1	3 (S_3S_{F10})	12 (S_3S_{11F})¶¶		1:1	10	6	1:1
4	D1		selfed	7 (S_3S_3)	13 (S_3S_{F11})	6 ($S_{F11}S_{F11}$)	1:2:1	25	5	3:1
D2† —1	D2(S_3S_{F11})	× S_1S_1		13 (S_1S_3)	15 (S_1S_{F11})		1:1			
2	D2	×$S_{F10}S_{F10}$		12 (S_3S_{F10})	9 ($S_{F10}S_{F11}$)		1:1			
3	$S_{F10}S_{F11}$	×	D2	14 (S_3S_{F10})	12 (S_3S_{F11})		1:1			
D4† —1	$S_{F10}S_{F11}$	×	D4(S_1S_{F11})	5 (S_1S_{F10})	12 (S_1S_{F11})		1:1			
D5† —1	D5(S_1S_{F10})	×	$S_{F10}S_{F11}$	18 (S_1S_{F11})§§	0 ($S_{F10}S_{F11}$)		1:1			
D6† —1	D6(S_3S_{F11})	×	S_1S_1	13 (S_1S_{F11})		8 ($S_1S_3S_{F11}$)				
D7† —1	D7(S_1S_{F10})	×	$S_{F11}S_{F11}$	11 (S_1S_{F11})	3 ($S_{F10}S_{F11}$)	4 ($S_1S_{F10}S_{F11}$)**				

*Testers were normal diploid plants with white (pp) or pink (Pp) flowers. Brackets indicate reciprocal crosses.
†These are white flowered plants with no apparent 'transferred' flower colour factors.
‡Segregation expected if the transferred gene is heterozygous and behaves normally.
§Includes normal and 'transferred' red flowered plants.
¶Plant C2(S_1S_{F11}) apparently forms (S_1S_{F11}) as well as (S_1) and (S_{F11}) gametes and viability of different types of gametes vary between combinations.
‖Plant D6(S_3S_{F11}) apparently forms gametes (S_{F11}) and (S_3S_{F11}) only.
**Plant D7(S_1S_{F10}) apparently forms (S_1S_{F10}) as well as (S_1) and (S_{F10}) gametes.
††Highly significant excess of pink-flowered plants ($P < 0.01$). All other '1:1' segregations do not differ significantly from expectation but are homogeneous in direction. The overall data show a highly significant excess of pink-flowered progeny ($P < 0.01$).
‡‡Highly significant deficit of pink-flowered plants ($P < 0.01$). All other '3:1' segregations do not differ significantly from expectation.
§§One class carrying transferred S gene. Cross C1-1 shows a significant deficit of the transferred gene ($P < 0.05$). Cross D5-1 shows a highly significant excess of the transferred gene ($P < 0.01$). Other segregations do not differ significantly from expectation.
¶¶Both classes carrying the transferred S gene. Cross D1-3 shows a significant excess of the S_3S_{F11} combination. All other segregations do not differ significantly from expectation.
‖‖Contained certain number of abnormal plants with vegetative floral shoots ($C_{1/3}$—5 plants, $C_{2/2}$—8, $C_{2/4}$—2, $C_{4/2}$—2, $D_{4/1}$—3).

it has been possible to demonstrate that both substitution and addition have occurred, although substitution seems to be predominant. It should be noted, however, that the patterns of transfer seen in these experiments may not be strictly comparable with those seen in previous attempts to induce transformation in higher organisms with chemically extracted preparations of DNA and chromatin. Extraction procedures and consequent denaturation of chromatin[11] are avoided by the present method, and the exogenous chromatin is received by a single specialised type of cell.

There are certain important features of the present results for which there seems to be no reasonable explanation at present. The frequency of specific transfers of the flower colour and S genes (the only two genes investigated) is unaccountably high among the rare seeds produced. One might suggest that there is a selection for the S gene transfer, for the heterozygosity of the S complex which is involved in the physiology of reproduction may be instrumental in the induction of parthenogenetic diploidy. The same cannot be said to be true of the flower colour gene however. There is a lack of S gene transfers in families A and B (Table 1) compared with families C and D. The segregation data, particularly of plants C1, C2, D1, D5 and D7, suggest the existence of situations which may influence gametic selection in extreme and contradictory ways. Apparently, wide variability is inherent in this induced phenomenon.

Doy *et al.*[12] have coined the term 'transgenosis' to describe the "transfer of genetic information from one cell to

another followed by phenotypic expression". The present phenomenon in *Nicotiana* can be regarded as a specialised from of 'sexual transgenosis'. Although there are certain mysterious aspects of the gene transfer phenomenon observed in these experiments, these new techniques are theoretically and technically attractive.

I thank Mr R. J. Trott, Palmerston North Hospital, for his help in the irradiation of pollen and Mr G. B. Petterson for technical assistance.

K. K. PANDEY

Genetics Unit,
Grasslands Division, DSIR,
Palmerston North,
New Zealand

1 Brewbaker, J. L., and Natarajan, A. T., *Genetics*, 45, 699–704 (1960).
2 Pandey, K. K., *Nature*, 206, 792–795 (1965).
3 Pandey, K. K., *Heredity*, 22, 255–284 (1967).
4 Goodspeed, T. H., *The Genus* Nicotiana (Chronica Botanica, Waltham, Massachusetts, 1954).
5 Pandey, K. K., *Theoret. appl. Genet.*, 44, 199–205 (1974).
6 De Petrocellis, B., and Monroy, A., *Endeavour*, 33, 92–97 (1974).
7 Hess, D., *Z. Pflanzenphysiol.*, 60, 348–358 (1969).
8 Fox, A. S., Yoon, S. B., Duggleby, W. F., and Gelbart, W. M., in *Informative Molecules in Biological Systems* (edit. by Ledoux, L.), 313–326 (North Holland, Amsterdam, 1971).
9 Ledoux, L., Huart, R., and Jacobs, M., *Nature*, 249, 17–21 (1974).
10 Hess, D., *Z. Pflanzenphysiol.*, 68, 432–440 (1973).
11 Merril, C. R., and Stanbro, H., *Z. Pflanzenphysiol.*, 72, 371–388 (1974).
12 Doy, C. H., Gresshoff, P. M., and Rolfe, B. G., in *The Biochemistry of Gene Expression in Higher Organisms* (edit. by Pollak, J. K., and Lee, J. W.), 21–37 (Australian and New Zealand Book Co., Sydney, 1973).

Salt-dependent bacteriophage infecting *Halobacterium cutirubrum* and *H. halobium*

MOST molecular genetic mechanisms are sensitive to ionic strength. The isolation of phage for *Halobacterium* allows a study of genetic adaptation in the extremes of ionic strength. The usual requirement for low ion concentrations is reversed in *Halobacterium* which maintains intracellular KCl near saturation[1]. As a result, macromolecular structure may be altered sufficiently to favour genetic mechanisms that occur rarely or not at all in other organisms. The presence of a unique DNA density distribution[2] and RNA-dependent RNA-polymerase[3] in *Halobacterium* suggests that such alterations occur.

Specific ion requirements define, to a great extent, the ecological niche of halophilic species, providing an advantage in general studies of phage ecology. The nature of halophilic genetic adaptation has a bearing on the suitability of seawater evaporates as sites for biogenesis and evolution.

Halobacterium flourishes in salt saturated environments and requires 10–12% NaCl to remain viable[4]. Water samples bearing phage were collected from The Salt Ponds of Yallahs, Jamaica, where the salt concentration varies from about 10% to saturation, depending on rainfall. Enrichment cultures were prepared from 1 ml of crude sample incubated in halophile broth for five d at 37 °C with aeration followed by addition of 2 ml late stationary phase *H. cutirubrum* and 10 d of additional incubation. The resulting culture was shaken with 1 ml chloroform and the supernatant collected after low speed centrifugation. Phages were purified by picking isolated plaques several times in succession from plates showing only a single plaque with *H. cutirubrum* as indicator.

Only phages forming clear plaques were detected in enrichment cultures or by direct plating of a sample containing 20% salt collected when the halobacterial flora was returning after having been greatly reduced by rainwater. One of these was selected and designated Halophage Ja. 1. The host range of phage Ja. 1 includes strains of *H. cutirubrum* and *H. halobium* reported in Table 1. The frequency of colonies surviving high multiplicities of phage varies widely among sensitive strains and several resistant colonies showed altered pigmentation. A mixed population of phages forming clear and turbid plaques on *H. cutirubrum* was present in a second sample collected 5 months later after evaporation had resulted in salt saturation and a red halobacterial bloom.

For determination of nucleic acid content lysates of Ja. 1 were purified by differential centrifugation and filtration through Whatman No. 1 filters. Concentrated suspensions were dialysed into 0.2 M NaCl, 0.05 M MgSO₄, 0.01 M Tris (*p*H 7.4) before a final cycle of low and high speed centrifugation. This procedure removes nucleic acid adhering to heavy cell debris while phage viability is maintained. Purified suspensions containing 10^{12} phage per ml were dialysed into 0.1 M NaCl, producing an extremely viscous clear solution of disrupted phage and $<10^4$ viable phage per ml. Reaction of the disrupted phage with diphenylamine[5] showed 1.5×10^8D DNA are present for each viable unit before disruption. This amount of DNA slightly exceeds the estimated capacity of the phage head shown in Fig. 1. Further purification of phage Ja. 1 on preformed CsCl gradients gave a single, sharp band of viability at 1.55 g cm⁻³ containing proportionately the same amount of DNA per viable unit. The presence of double-stranded DNA is confirmed by specific immunological assay as is single stranded DNA after boiling for 5 min. Double
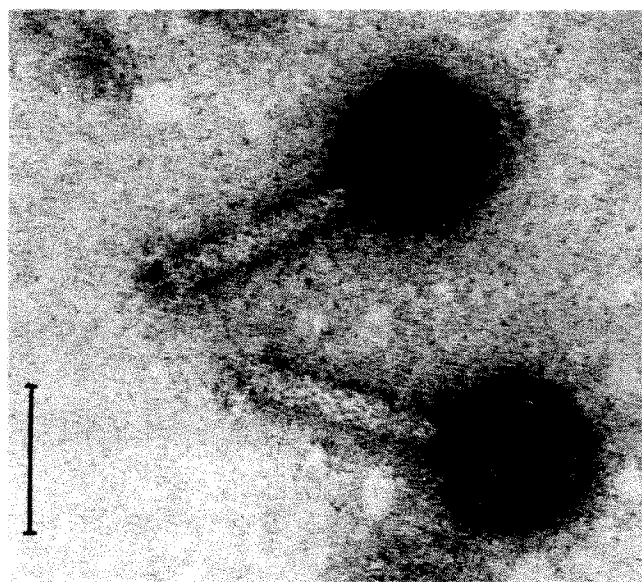
Fig. 1 Halophage Ja.1 suspensions in 0.2 M NaCl, 0.05 M MgSO₄ were fixed on Formvar–carbon-coated grids in glutaraldehyde vapours for 10 s and negatively stained with uranyl acetate. The bar represents a distance of 0.1 μm. The phages pictured here are of the same morphological group although larger than particles observed in crude preparations of *H. salinarium* flagella[11].

stranded RNA, RNA–DNA hybrids and single stranded DNA before heat treatment are not detected with corresponding antisera (refs 6 and 7 and A.C.W. and B.D.S., unpublished).

Table 1 Host range of Ja.1

	Host organism	Source	Plating efficiency
1.	*H. cutirubrum*	Laboratory strain	1.0
2.	*H. cutirubrum*	S. T. Bayley	0.2
3.	*H. cutirubrum*	L. I. Hochstein	$<2 \times 10^{-9}$
4.	*H. halobium*, Brown	L. I. Hochstein	0.6
5.	*H. halobium*, Delft	L. I. Hochstein	0.01
6.	*H. halobium*, Delft	K. Eimhjellen	$<2 \times 10^{-9}$
7.	Utah 18*	L. I. Hochstein	0.4
8.	Utah*	L. I. Hochstein	$<2 \times 10^{-9}$
9.	AR1, Lanyi†	L. I. Hochstein	$<2 \times 10^{-9}$
10.	*Sarcina morrhuae*	L. I. Hochstein	$<2 \times 10^{-9}$
11.	*Sarcina littoralis*	K. Eimhjellen	$<2 \times 10^{-9}$
12.	*H. salinarium*	K. Eimhjellen	$<2 \times 10^{-9}$

Host organisms were grown at 37 °C with shaking in halophile broth containing 9 parts salt solution (20% NaCl, 2% KCl, 2% MgSO₄), and 1 part nutrient solution (4% Difco yeast extract, 4% Difco casamino acids, 3% sodium citrate, 20% NaCl, 2% KCl, 2% MgSO₄). Agar for agar plates was prepared by mixing 500 ml agar salts solution (25% NaCl, 2.5% KCl, 2.5% MgSO₄), 60 ml nutrient solution and 8 g Difco agar in 100 ml distilled water. All solutions were autoclaved at 121 °C before use. Agar for overlays was prepared by mixing 40 ml salt solution, 50 ml distilled water, 0.4 g ion agar No. 2 and autoclaving for 15 min at 121 °C. 10 ml nutrient solution was added to the hot solution and 2.5-ml aliquots dispensed in small tubes. These were allowed to solidify, melted and tempered to 60 °C as needed. Host bacteria were collected by centrifugation in mid log phase and concentrated 20 times in a medium containing 9 parts agar salts solution and 1 part nutrient solution and held at 0–4 °C. Five drops of the host suspension was added to the overlay before the addition of phage. Plates were incubated at 38 °C and plaques appeared in less than 24 h. Lysates of phage Ja.1 containing $2–3 \times 10^{10}$ phage ml⁻¹ were produced by diluting 30 ml of a late stationary phase culture of *H. cutirubrum* into 200 ml halophile medium, infecting with eight 25-h plaques and shaking vigorously, until lysis was complete, about 24 h at 37 °C. Phage dilutions were made into complete halophile medium or a similar high salt nutrient solution.
*Isolated from Great Salt Lake, USA.
†Isolated from salterns near San Francisco, USA.

Table 2 Phage Ja.1 survival in ionic solutions

Solution	Fraction surviving		
	30min	24h	96h
3.5 M NaCl, 0.05 M MgSO$_4$	1.0	1.0	1.0
2 M NaCl	0.8	0.6	0.3
1 M NaCl	0.5	10^{-5}	< 10^{-7}
0.2 M NaCl	< 10^{-7}	—	—
2 M KCl	0.8	0.6	0.5
1 M KCl	0.7	0.1	0.01
0.2 KCl	< 10^{-7}	—	—
0.02 M MgSO$_4$	0.7	0.6	0.4

A plate stock containing 2×10^{10} phage Ja. 1 ml^{-1} diluted 1:200 into various ionic solutions at 4 °C each containing 0.01 M Tris buffer (pH 7.4). Phage titres were determined at intervals after dilution. Relative rates of inactivation are similar at 37 °C.

The single step growth function for phage Ja. 1 infection of *H. cutirubrum* is shown in Fig. 2. Only 25% of the input phage are present as chloroform sensitive infective centres after a 30-min adsorption period and 25% are present as chloroform resistant, presumably unadsorbed particles. After a 2-h eclipse, maturation of phage begins and is complete in 6 h which is the normal generation time for the bacteria alone growing in the same conditions. The apparent average burst size is 140. Lysis begins only after maturation is complete and occurs over a 3–4-h period. The abrupt cessation of phage maturation before lysis suggests that a specific function limits the number of particles produced in each cell. Phage T4, a model of lytic development, continues to mature particles in an infected cell until lysis is achieved[8]. Inefficient adsorption, limited maturation, and delayed lysis in Ja. 1 infection may favour an equilibrium between phage and host populations in a closed natural habitat. These

Fig. 2 A *H. cutirubrum* culture in log phase containing 1.5×10^8 viable cells ml^{-1} was infected with 1.2×10^7 plaque forming units ml^{-1} from a plate stock of Ja.1. After a 30-min absorption period the culture was diluted times 6×10^3 into fresh medium at 37 °C. Aliquots of the diluted culture were titred at intervals before and after adding a drop of chloroform. Chloroform lyses the cells but does not reduce the titre of completed phage particles. Plaque titre in the absence of chloroform is plotted minus the number of complete, chloroform resistant, particles present at early times to derive the titre of cells producing phage plus the titre of phage released from these in the culture, ○. The titres obtained after chloroform treatment, representing all complete phage present is plotted, ●.
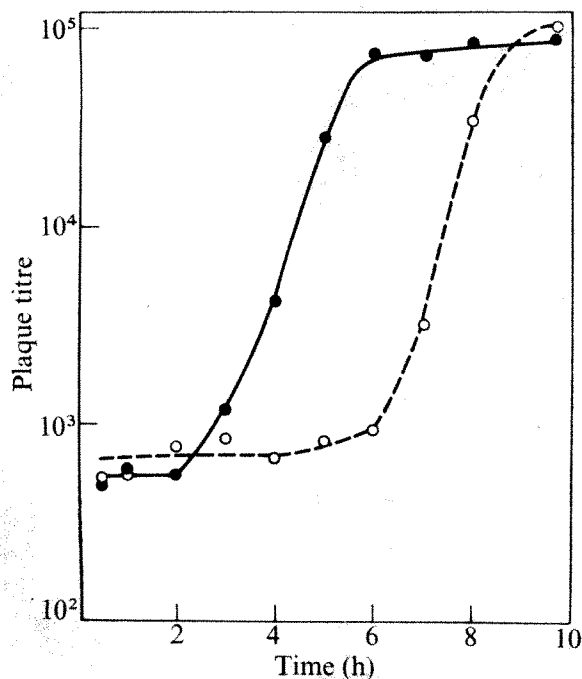
functions could not benefit T4 whose host, *Escherichia coli*, cannot establish a stable population outside the gut.

Viability in phage Ja. 1 is preserved only in solutions of high ionic strength. The ions and concentrations required to protect Ja. 1 reflect intracellular ionic conditions in *H. cutirubrum* and are similar to those that favour the stability of halophilic enzymes[9]. With few exceptions, enzymes isolated from *Halobacterium* require 1 M to 4 M concentrations of monovalent cations for optimum stability and activity. In most instances K$^+$ is more efficient than Na$^+$ in filling this requirement and 0.05 M Mg^{2+} often reduces or eliminates a requirement for monovalent ions. Table 2 shows that 1 M KCl affords Ja. 1 several orders of magnitude greater protection against irreversible loss of viability than does 1 M NaCl and a high degree of stability is conferred by 0.02 M Mg^{2+}. In contrast, host viability and growth is strictly dependent on Na$^+$ concentrations exceeding 2 M and the presence of other ions does not materially reduce this dependence[4]. Phage survival in 0.02 M Mg^{2+} suggests that the phage survives and may even function in more moderate environments than does its host species. At the opposite extreme, 10^8 phage Ja. 1 are destroyed by drying for 2 d at 22 °C and moderate humidity although the host species survives for many years in salt crystals[10].

Contrary to the finding for Ja. 1, marine phage[12] survives better in NaCl than in KCl although marine bacteria often have high intracellular KCl[1]. Sparse host populations in the marine environment may cause phage to adapt to extracellular conditions while seawater evaporates, such as The Salt Ponds of Yallahs, support extremely dense microbial populations. Further study of the sequence of appearance, ionic niche requirements, and cellular genetic roles of halophages may reveal their ecological function in the environmentally induced growth cycles of the halophilic flora.

A. C. WAIS*
M. KON
R. E. MACDONALD

Section of Biochemistry, Molecular and Cell Biology,
Cornell University,
Ithaca, New York

B. D. STOLLAR

Tufts University School of Medicine,
Boston, Massachusetts

1 Christian, J. H. B., and Waltho, J. A., *Biochim. biophys. Acta*, **65**, 506–508 (1962).
2 Moore, R. L., and McCarthy, B. J., *J. Bact.*, **99**, 248–254; 255–262 (1969).
3 Louis, B. G., and Fitt, P. S., *Biochem. J.*, **128**, 755–762 (1972).
4 Larsen, H., *Adv. Microbiol. Physiol.*, **1**, 39–90 (1967).
5 Burton, K., in *Methods in Enzymology*, **12**, Part B (edit. by Grossman, L., and Moldave, K.), 163–165 (Academic, New York and London, 1968).
6 Stollar, B. D., *Science*, **169**, 609–611 (1970).
7 Stollar, B. D., and Sandberg, A. L., *J. Immun.*, **96**, 755–763 (1966).
8 Wais, A. C., and Goldberg, E. B., *Virology*, **55**, 397–399 (1973).
9 Lanyi, Y. K., *Bact. Rev.*, **38**, 272–290 (1974).
10 Larsen, H., in *The Bacteria*, **4** (edit. by Gunsalus, I. C., and Stanier, R. Y.), 279–342 (Academic, New York and London, 1962).
11 Torsvic, T., and Dundas, I. D., *Nature*, **248**, 680–681 (1974).
12 Keynan, A., Nealson, K., Sideropoulos, H., and Hastings, J. W., *J. Virol.*, **14**, 333–340 (1974).

Morphology and biochemistry of small, intensely fluorescent cells of sympathetic ganglia

NEURAL transmission in sympathetic ganglia has been regarded as an automatic relay involving only pre- and postganglionic neurones. It is now agreed, however, that the small, intensely fluorescent cells — at least some of

which are interneurones[1,2] — modulate transmission in the superior cervical ganglion. Greengard *et al.*[3-6] have documented the role of the ganglionic interneurone in modulating ganglionic transmission. Dopamine released from an interneurone binds to a receptor on the surface of the ganglionic neurone, activating an adenylate cyclase. The increased production of cyclic AMP leads to hyperpolarisation of the ganglionic neurone by a mechanism which is still obscure.

Studies (T.C. and T.H.W., unpublished) have shown that the morphology of the sympathetic interneurone is considerably more complex and shows more species diversity than is sometimes appreciated, which raises the possibility of major species differences in its biochemistry and in the mechanisms whereby it modulates transmission in the ganglion. We have demonstrated that the biochemistry of the interaction between the ganglionic interneurone and the principal ganglionic neurone is different in the cat from that in the cow.

Figure 1 shows the effect of dopamine on the production of cyclic AMP in bovine and feline superior cervical ganglia incubated *in vitro*. In the bovine ganglion there is a marked increase in the amount of cyclic AMP produced by even a 1 μM concentration of dopamine and, moreover, the response obtained is proportional to log dopamine concentration (is dose dependent[7]). Our work in the bovine ganglion confirms the findings of Kebabian and Greengard[8]. In the feline ganglion (Fig. 1) the situation is different. The cat ganglion proved relatively unresponsive to dopamine stimulation and, within the range of concentrations used, the cyclic AMP response was not dose dependent. We also determined the effect of *l*-noradrenaline on the cat superior cervical ganglion incubated *in vitro* to make certain that we were not attempting to stimulate with the incorrect neuro-transmitter. This compound was ineffective even at concentrations of 100 μmol l^{-1} (ref. 9). Thus, the feline ganglion contains an adenylate cyclase which is relatively unresponsive to stimulation by dopamine or *l*-noradrenaline.

These findings led us to search for a morphological basis for these biochemical differences. Do the ganglionic interneurones of cat superior cervical ganglion differ from those of the bovine ganglion in a manner which could account for the differences in the observed responses to dopamine? Morphological studies of bovine and feline superior cervical ganglia were undertaken using the method of Grillo *et al.*[10], as modified by T.C. and T.H.W. (unpublished). This method enables the preparation of sections for fluorescence microscopy and subsequent examination of the same material by electron microscopy. In both species two types

Fig. 2 Histochemical preparations demonstrating catecholamine fluorescence. *a*, Single small intensely fluorescent cell (type I) is seen in close apposition to a ganglionic neurone (bovine superior cervical ganglion). Single arrow, a principal ganglionic neurone; double arrow, a ganglionic interneurone with a process. *b*, Cluster of small fluorescent cells (type II) in the capsular connective tissue of the feline superior cervical ganglion. *c*, Capillary lumen. Bar represents 20 μm.

of small, intensely fluorescent cells were distinguished. Type I (Fig. 2*a*) has long processes which run in close apposition to ganglion cells. This is regarded as the type of cell shown by Williams[1,2] to be an interneurone in the rat: the presence of this type I ganglionic interneurone is crucial to the Greengard hypothesis. The type II cell (Fig. 2*b*) is located in the interstitial or subcapsular portions of

Fig. 3 Ultrastructure of type II small, intensely fluorescent cells in cat and cow. *a*, Cat (perfusion-fixed for fluorescence microscopy and electron microscopy) showing characteristic dense granules (1,400–1,800 Å). *b*, Cow (immersion-fixed) also exhibiting dense granules. Hollow centres of these granules are presumed to be a fixation artefact. Bar represents 1 μm.

Fig. 1 Effect of dopamine on cyclic AMP levels of superior cervical ganglia incubated *in vitro* at 37 °C. ○, Bovine ganglia; ●, feline ganglia. Control values: bovine ganglia 28.8±2.18 pmol cyclic AMP per mg protein; feline ganglia 26.9±5.7 pmol per mg protein. Each point is the mean of four to six samples ± s.e. mean. All samples were incubated in Eagle's MEM containing 1 mM theophylline. Cyclic AMP assays were carried out according to Gilman[12].

the ganglion, and has shorter processes which end in close relation to blood vessels[11]. Type II cells from both feline and bovine superior cervical ganglia exhibited similar ultrastructural features (Fig. 3a and b).

Quantitative studies of the total small, intensely fluorescent cell population in each species, using fluorescence histochemistry, showed that the bovine superior cervical ganglion contains a total population of 6,480 small, intensely fluorescent cells per ganglion (5.2 cells per mg wet tissue), and the feline ganglion contains 104 cells (4.1 cells per mg wet tissue). Thus the numbers per mg tissue are not very different. The cow ganglia contained 24% type I ganglionic interneurones, the remaining 76% being classified as type II. Of all the small, fluorescent cells examined in the feline superior cervical ganglion, only 2% were type I; the rest were type II cells. Thus type I cells are common in the cow, whereas they are virtually absent in the cat. As the dopamine receptor–adenylate cyclase complex is located on the postsynaptic membrane of the synapse between the interneurone and the principal ganglionic neurone, the scarcity of ganglionic interneurones (type I small, intensely fluorescent cells) in the cat ganglion may account for the meagre response to dopamine in this species.

These results raise the question: are all small, intensely fluorescent cells interneurones? The answer may well be no. There exist at least two types of small, intensely fluorescent cells, one of which (type I) can be compared with the classical interneurone first described in the rat by Williams[1,2]. The other (type II) is probably not an interneurone and is (at least in the bovine and feline superior cervical ganglion) associated with blood vessels, leading one to suspect a functional association with these vessels.

In summary, there are two main types of small, intensely fluorescent cell in the superior cervical sympathetic ganglion. We have noted a virtual absence of the type I cell (comparable with the interneurone in the rat) in the cat. Also absent or much reduced in the cat is the dopamine-sensitive adenylate cyclase receptor mechanism involved in neural transmission in other species. We therefore infer that the type I cell is a ganglionic interneurone capable of activating this receptor mechanism. Type II remains an enigma.

T. H. WILLIAMS
A. C. BLACK, JR
T. CHIBA
R. C. BHALLA

Department of Anatomy,
University of Iowa,
Iowa City, Iowa 52242

1 Williams, T. H., *Nature*, **214**, 309–310 (1967).
2 Williams, T. H., and Palay, S. L., *Brain Res.*, **15**, 17–34 (1969).
3 Greengard, P., McAfee, D. A., and Kebabian, J. W., in *Advances in Cyclic Nucleotide Research*, **1** (edit. by Greengard, P., Robison, G. A., and Paoletti, R.), 337–355 (Raven, New York, 1972).
4 Greengard, P., Kebabian, J. W., and McAfee, D. A., in *Proc. fifth Int. Congr. Pharmac., San Francisco 1972*, **5**, 207–217 (1973).
5 Greengard, P., and Kebabian, J. W., *Fedn Proc.*, **33**, 1059–1067 (1974).
6 McAfee, D. A., and Greengard, P., *Science*, **178**, 310–312 (1972).
7 Black, A. C., Jr, Bhalla, R. C., and Williams, T. H., *Progr. Soc. Neurosci., fourth Ann. Meeting, St. Louis, Missouri*, Abstract 72 (1974).
8 Kebabian, J. W., and Greengard, P., *Science*, **174**, 1346–1349 (1971).
9 Black, A. C., Jr, Bhalla, R. C., and Williams, T. H., *Anat. Rec.*, **178**, 311 (1974).
10 Grillo, M. A., Jacobs, L., and Comroe, J. H., Jr, *J. comp. Neurol.*, **153**, 1–14 (1974).
11 Chiba, T., Black, A. C., Jr, and Williams, T. H., *Anat. Rec.*, **181**, 331 (1975).
12 Gilman, Alfred G., in *Advances in Cyclic Nucleotide Research*, **2** (edit. by Greengard, P., Robison, G. A., and Paoletti, R.), 9–24 (Raven, New York, 1972).

*p*H-sensitive cells at ventro–lateral surface of rat medulla oblongata

MARKED effects of *p*H changes in the cerebrospinal fluid (CSF) on ventilation have been confirmed by a number of investigators[1]. Consistent augmentation of respiration by applying acid fluids

to specific regions on the ventro–lateral surface of the cat medulla oblongata[2,3] has been shown, and similar results were observed with the brain stem of the rat[4]. The chemosensitive nature of these regions proposed from the above experiments has been substantiated by electrophysiological studies using an extracellular recording technique[5,6]. Movements of the brain as a result of respiration and cardiac activity, however, make it difficult to obtain intracellular recordings of nerve activity in these structures. Therefore, thin brain slices were used *in vitro* in the present experiment.

The brain stem of the rat at the level from the 1st cervical nerve to the roots of the vagal and glossopharyngeal nerves was excised under intraperitoneal chloralose (60 mg kg^{-1})–urethane (250 mg kg^{-1}) anaesthesia. A thin brain slice (0.3–0.5 mm thick) was dissected from either side of the ventral surface of the medulla as described previously[7]. Preparations were placed on a nylon mesh in the incubation chamber, ventral surface upwards, and perfused continuously with mock CSF at various *p*H (7.0–7.8) at 36 °C. Normal mock CSF (*p*H 7.4) had the following composition (mM): NaCl, 125; NaHCO$_3$, 25; KCl, 3.5; CaCl$_2$, 1.3; MgCl$_2$, 1.1; NaH$_2$PO$_4$, 0.51; urea, 2.2; and glucose, 3.4. A high or low *p*H solution was obtained by altering the NaHCO$_3$ concentration with constant P_{CO_2}. Solutions were equilibrated by a gas mixture containing 5.6% CO$_2$ in oxygen (P_{CO_2} 42 mmHg). In another series of experiments the P_{CO_2} was varied from 18 to 90 mmHg at constant *p*H. Transmembrane potentials were recorded using glass micropipettes filled with 3 M KCl having a resistance of 20–30 M Ω.

After 30 min incubation, a microelectrode was inserted into various parts of the surface layer (up to about 200 μm deep) and negative potentials from -50 to -75 mV, which indicated cell membrane activity, could be recorded at the regions lateral to the pyramid. The mean ($\pm$s.e.) membrane potential was -62 ± 2 mV at *p*H 7.4 and no significant differences in the membrane potential were seen in cells located at different portions of the ventral medulla. When the *p*H of the perfusing solution was altered from 7.0 to 7.8, however, cells located at the area medial to the root of XIIth cranial nerve, just lateral to the pyramid, showed apparent potential changes. Figure 1a shows a continuous recording of membrane potential from a single cell of this area. The membrane potential of this cell was about -78 mV at *p*H 7.8 and was depolarised gradually to about -58 mV by application of a low *p*H (7.0) solution. The average membrane potential of cells in this area was -55 and -75 mV at *p*H 7.0 and 7.8, respectively. We were unable

Fig. 1 Effect of changes in *p*H of perfusing solution on membrane potential of cells at the surface layer of the rat ventral medulla *in vitro*. a, Record from a single cell at the area medial to the XIIth cranial nerve (Loeschcke's area). Note the significant changes in potential with changes in *p*H. b, Record from a single cell at 1 mm cranial to the root of XIIth cranial nerve and 1 mm lateral to the pyramid. In both a and b *p*H was changed with constant P_{CO_2} (42 mmHg). MP, Transmembrane potential; XII, XIIth cranial nerve; C$_1$, 1st cervical nerve. Asterisk in schematic representation of the ventral medulla represents site at which potential was recorded. Bar, 1 min.
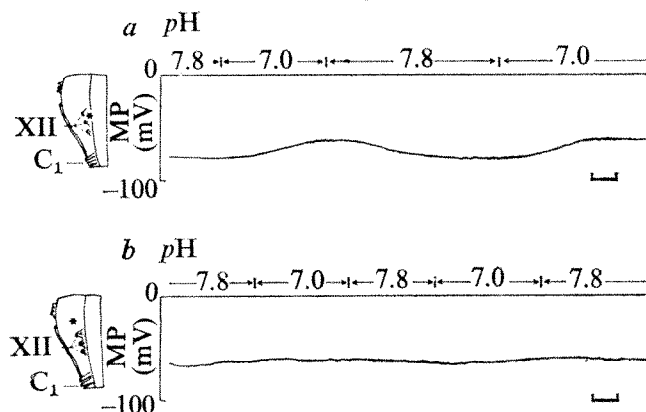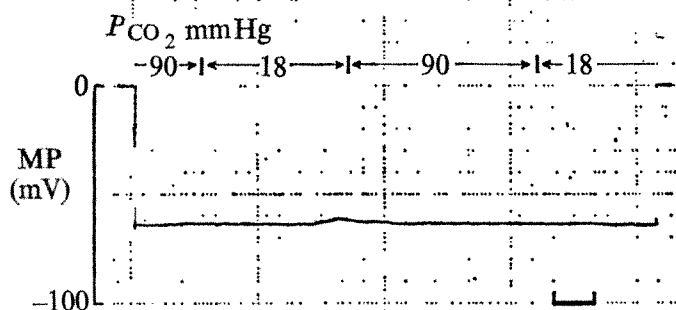
Fig. 2 Effect of changes in P_{CO_2} of perfusing solution on membrane potential of a cell at the area medial to the root of XIIth cranial nerve *in vitro*. pH was kept constant at 7.4, adjusting $NaHCO_3$ concentration. MP, Transmembrane potential. Bar, 1 min.

to record apparent spontaneous discharges from these cells during depolarisation when a low pH solution was applied. The membrane potential of cells in other areas did not change with alteration in pH of the perfusing solution, which indicates that cells have no sensitivity to changes in the hydrogen ion concentration of the extracellular fluid (Fig. 1b). On the other hand, when the P_{CO_2} of the perfusing solution was altered, without altering the pH by adjusting the $NaHCO_3$ concentration, membrane potentials of cells of all the surface area remained constant.

Figure 2 was obtained from a single cell located at the root of XIIth cranial nerve. In this case the membrane potential was about -64 mV at pH 7.4 with P_{CO_2} 42 mmHg and the potential was maintained as the P_{CO_2} was changed from 90 to 18 mmHg. Therefore, cells located at the area medial to the roof of XIIth cranial nerve (Loeschcke's area) seem to be characterised by being sensitive specifically to extracellular pH and not to P_{CO_2}. The same characteristics of unique pH sensitivity have also been demonstrated by *in vivo* studies[3,5,6] in which ventilation and neuronal discharges recorded from the ventral medulla were observed with the same perfusion experiments.

The site of localisation of pH-sensitive cells is in accord with the area at which the application of low pH solution causes the highest and most rapid augmentation of respiration in the cat *in vivo*[1,3]. Other areas which have been responsible for the respiratory chemosensitivity to changes in CSF pH have been located at the surface of the ventral medulla near the roots of the vagal and glossopharyngeal nerves (Mitchell's area)[1]. In the present experiment, however, there was no observed change in potentials recorded from the cells of this area with changes in pH or P_{CO_2}. It is suggested that cells located in deeper (more than 200 μm) layers of this area may be sensitive to pH (ref. 8).

pH or P_{CO_2}-sensitive cells have been reported in the abdominal ganglion of the marine mollusc *Aplysia*[9,10], and have also been found in cortical and spinal neurones of the rat[11,12]. In the present experiment pH-sensitive cells were shown to exist in the specific region of the medulla which has been assumed to be involved in the chemical regulation of respiration[1]. Whether this cell will exhibit spontaneous activity *in vivo* and be responsible for driving the ventilation or not, is not yet known. It is possible, however, that respiratory responses to changes in the pH of the CSF or brain extracellular fluid, and to changes in acid–base status of blood, may partly be initiated through this cell.

Y. Fukuda
Y. Honda

Department of Physiology,
Chiba University School of Medicine,
Chiba, Japan

1 Loeschcke, H. H., in *Respiratory Physiology* (edit. by Widdcome, J. G.), 167 (Butterworths, London, 1974).
2 Mitchell, R. A., Loeschcke, H. H., Massion, W. H., and Severinghaus, J. W., *J. appl. Physiol.*, 18, 523 (1963).
3 Schläfke, M. E., See, W. R., and Loeschcke, H. H., *Resp. Physiol.*, 10, 198 (1970).
4 Hori, T., Roth, G. I., and Yamamoto, W. S., *J. appl. physiol.*, 28, 721 (1970).
5 Schläfke, M. E., See, W. R., Herker, A., Luttmann, A., and Loeschcke, H. H., *Pflügers Arch.*, 347, R38 (1974).
6 Schläfke, M. E., See, W. R., and Kille, J. F., *Proc. Int. Union physiol. Sci.* (XXVI), abstract 269 (1974).
7 Yamamoto, C., and McIlwain, H., *J. Neurochem.*, 13, 1333 (1966).
8 Dermietzel, R., in *Acid–base Homeostasis of Brain Extracellular Fluid* (Abstract of symposium, Bochum, 1975).
9 Chalazonitis, N., *Ann. N.Y. Acad. Sci.*, 109, 451 (1963).
10 Brown, A. M., and Berman, P. R., *J. gen. Physiol.*, 56, 543 (1970).
11 Speckmann, E. J., and Caspers, H., *Pflügers Arch.*, 310, 235 (1969).
12 Speckmann, E. J., Caspers, H., and Sokolov, W., *Pflügers Arch.*, 319, 122 (1970).

BCG suppresses growth and metastasis of hydatid infections

THE asexually proliferating larvae of the cestode *Echinococcus multilocularis* have an intriguing resemblance to malignant tumours. Indeed, until 1856 when Virchow demonstrated the parasitic nature of these cyst masses in man, such lesions were commonly diagnosed as carcinomas of the liver[1]. In the laboratory, infections with this parasite can be propagated vegetatively by transplanting the metacestode into the peritoneal cavity of susceptible rodent intermediate hosts[2]. The protoscolices transform into small cysts which grow and metastasise by peripheral herniation and the detachment of minute vesicles[3].

Macrophages from donors which have been inoculated with microorganisms such as *Besnoitia, Toxoplasma* or BCG can nonspecifically destroy tumour cells *in vitro*[4-6]. BCG has been successfully used in the active, nonspecific immunotherapy of malignant tumours *in vivo*[7,8] and we report here that this same treatment may also be effective in the immunoprophylaxis and immunotherapy of *E. multilocularis* infections.

Pretreatment of the experimental cotton rat host (*Sigmodon hispidus*) with 26.4×10^8 colony-forming units (CFU) of BCG suppressed the growth and metastasis of an intraperitoneal inoculum of 200 protoscolices (Table 1). Thus, autopsy 50 d after inoculation revealed that only two of the four BCG-treated cotton rats of this group had developed echinococcosis. These two infected animals bore cyst masses weighing 0.5 g and 1.5 g which were composed of one and two foci respectively. In contrast, the four untreated control animals bore an average hydatid cyst weight of 14 g ($P < 0.01$) composed of an average of 40.5 distinct foci ($P < 0.005$).

BCG treatment 2 weeks after intraperitoneal inoculation of 200 protoscolices did not limit the establishment and growth of the hydatid infection. All animals within this group developed infections the weights of which were indistinguishable from controls ($P > 0.10$). BCG treatment after inoculation did, however, severely suppress the number of parasitic foci of infection which developed from the inoculum. Whereas controls bore an average of 40.5 foci of infection, treated animals bore only 4.8 ($P < 0.005$); the number of foci were also significantly greater ($P < 0.025$) in this group than in that treated before infection. The severity of this suppression is underlined by the fact that one protoscolex can give rise to more than 20 foci of infection by metastasis in cotton rats[9].

Our study indicates that prior BCG treatment suppresses the growth and metastasis of an intraperitoneal infection with *E. multilocularis*. BCG treatment of established infections, on the other hand, only limits the parasite metastases, whereas growth (assessed by the total weight of the cyst biomass) seems to proceed normally. These findings further emphasise the striking similarities between this parasitic infection and the growth and proliferation of malignant tumours. Baldwin and Pimm[10] have reported that pulmonary metastases from primary rat hepatomata can be similarly restricted by BCG treatment. They report that, again, this treatment had no influence on the occurrence or growth

Table 1 Effect of BCG treatment on growth and metastasis of *E. multilocularis*

Untreated infected controls		BCG treatment 1 week before inoculation		BCG treatment 2 weeks after inoculation	
Total weight of cyst mass (g)	No. of foci	Total weight of cyst mass (g)	No. of foci	Total weight of cyst mass (g)	No. of foci
10.5	47	1.5	2	8.8	3
9.0	29	0.0	0	12.0	3
12.6	36	0.5	1	20.0	8
23.9	50	0.0	0	12.7	5
Mean 14.0	40.5	0.5	0.8	13.4	4.8

Twelve 5-week-old cotton rats were each inoculated with 200 protoscolices of *E. multilocularis*, and autopsied 50 d later for hydatid cyst masses. Eight of these animals had been treated with an intraperitoneal injection of 26.4×10^6 CFU of lyophilised BCG (lot no. 1707-6; Institut de Microbiologie et d'Hygiène de Montréal) in 0.5 ml distilled water. Four of these latter animals had been treated 1 week previously and the other four rats 2 weeks after inoculation with the parasite.

rates of primary tumours (assessed by the survival of experimental animals).

The treatment dose of 26.4×10^6 CFU of BCG was tolerated well by our animals although all cotton rats treated before or after inoculation with the parasite developed small intraperitoneal granulomatous lesions.

We are therefore continuing the search for a treatment regimen that will maximise the immunoprophylactic and immunotherapeutic effects of BCG in hydatid disease without the production of granulomas.

M. E. RAU
C. E. TANNER

Institute of Parasitology,
McGill University, Macdonald College,
Quebec, Canada H0A 1C0

1 Virchow, R., *Verh. Phys.-Med. Ges. Würzburg*, 6, 84–95 (1856).
2 Lubinsky, G., *Can. J. Zool.*, 38, 149–151 (1960).
3 Webster, G. A., and Cameron, T. W. M., *Can. J. Zool.*, 34, 877–891 (1961).
4 Hibbs, J. B., Jr, Lambert, L. H., Jr, and Remington, J. S., *J. Infect. Dis.*, 129, 587–592 (1971).
5 Hibbs, J. B., Jr, Lambert, L. H., Jr, and Remington, J. S., *Nature new Biol.*, 235, 48–50 (1972).
6 Cleveland, R. P., Meltzer, M. S., and Zbar, B., *J. natn. Cancer Inst.*, 52, 1887–1895 (1974).
7 Bluming, A. Z., Vogel, C. L., Ziegler, J. L., Mody, N., and Kamya, G., *Ann. intern. Med.*, 76, 405–411 (1972).
8 Gutterman, J. U., Mavligit, G., McBride, C., Frei, E., and Hersh, E. M., *Lancet*, i, 1208–1212 (1973).
9 Rau, M. E., and Tanner, C. E., *Can. J. Zool.*, 51, 55–59 (1973).
10 Baldwin, R. W., and Pimm, M. V., *Br. J. Cancer*, 30, 473–476 (1974).

Host growth induced by genetic tumour grafts

SPONTANEOUS tumours in *Nicotiana* were first reported by Kostoff[1] and are called genetic tumours[2]. They arise on certain interspecific tobacco hybrids[2], and it has been proposed that certain genes when appropriately combined in a hybrid promote the development of tumours[3,4]. Though in bacterially induced crown gall tumours the tumour state is transmissible from a tumour cell to a normal cell in the absence of viable bacteria[5,6], efforts to demonstrate an ability of genetic tumours to induce tumour development across a graft union have failed[2]. Attempts to graft *Nicotiana glauca* × *Nicotiana langsdorffi* hybrid tumour tissues from sterile culture to non-parental species of *Nicotiana* were unsuccessful because the grafts died[7,8]. Here we report the successful grafting of *N. glauca* × *N. langsdorffii* amphidiploid (GGLL) tissue from sterile culture on to *N. tabacum* cv. Wisconsin 38. Within a month of grafting, the graft (GGLL tumour tissue) induced the development of host (*N. tabacum*) shoots in wound sites and some grafts became chimaeral.

We shall describe one of the three separate grafting experiments we performed. Fifteen grafts were made, 11

produced GGLL tumours, and four grafts induced the development of one or more host shoots within a month of grafting. In one graft the shoot came from the side of the tumour, in another from the tumour region (Fig. 1*a*), and in the other two cases shoots came from the wound area where the graft did not take. Sussex *et al.*[9] never observed shoot development from comparably wounded internodal areas of the same strain of *N. tabacum*, and we have wounded plants and have grafted numerous pieces of *N. tabacum* tissues into *N. tabacum* plants without ever observing shoot development from an internodal wound.

One shoot growing out of a graft (Fig. 1*b*) was rooted and the genotype of its tissue layers was determined. Its morphology was *N. tabacum*, its seed germinated into *N. tabacum* plants, and its pith regenerated in culture to produce *N. tabacum* plants. Thus the three layers in its shoot apical meristem, T_1, T_2, and C, were genetically *N. tabacum*. In another instance a sectorial chimaera was produced (Fig. 1*c*). As this shoot grew, the GGLL sector was eliminated, but by removing the apex, we forced an axillary bud from the GGLL sector to develop. This bud produced a shoot which was also a mixture of graft and host cells as indicated by tumour production, leaves of intermediate shape, and flowers with morphological characters of both GGLL and *N. tabacum*.

Bacteria-free crown gall tumour cells are reported to produce or contain one or more substances which cause neighbouring cells to become tumorous[5,6]. As indicated from previous work[2,7,8] and from the results presented here, it is unlikely that genetic tumour cells can influence cells with a non-tumorous genotype to become tumorous. The genetic tumour graft does, however, influence host cells to divide and organise shoot meristems Since host shoots emerged from graft tissue, the tumours must in some cases be chimaeral, and this assumption is strengthened by the observation of one chimaeral shoot. Although it is possible that tumorous host cells exist among the graft cells, it is more plausible that host cells are induced to divide under the influence of graft growth regulators of the auxin and cytokinin types. That is, GGLL tumours produce auxin and cytokinin[10], and when *N. tabacum* plants are wounded and the wound exposed to these growth regulators, shoot meristems develop (C.N.M., unpublished). In addition, no growth is observed on the host side of the graft union whereas cell division and growth occur on the host side of the graft union during tumour induction with sterile bacterial tumour tissue[5,6].

Although crown gall tumours and genetic tumours exhibit similar hormonal autonomy in tissue culture and retention of abnormal growth on grafting[11], these results suggest there is a fundamental difference in their capacity to influence non-tumorous cells of a host into which they are grafted. Genetic tumours arise spontaneously or appear following injury or external hormone application[2,12]. In the case of crown gall, both injury and the presence of
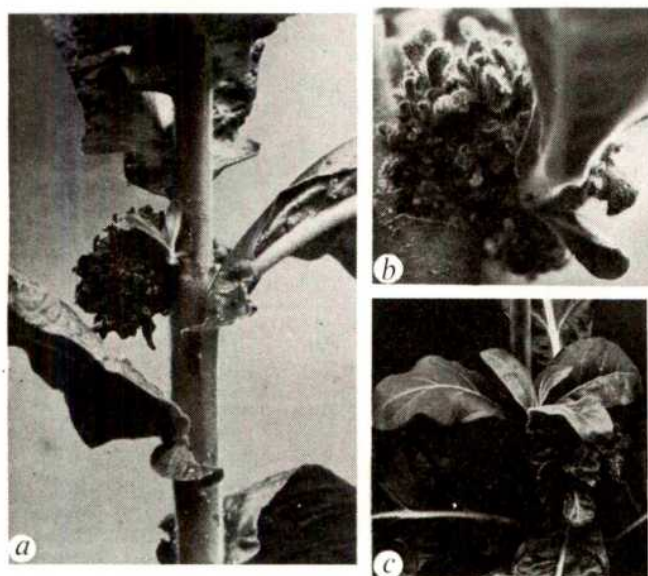
Fig. 1 GGLL tumour tissue was grown in sterile culture on Linsmaier and Skoog medium[15] without hormonal supplement. Small pieces (0.075 to 1.0 cm²) of GGLL tumour were grafted to the top or into a V-shaped lateral notch in the stem of greenhouse grown *N. tabacum* plants. Before grafting, plants were decapitated, and graft tissue was always placed in an internodal area. Plants were used after bolting but before anthesis (flowering) and axillary buds were usually removed as they grew out. *a, b, N. tabacum* shoots growing out of grafted tumour tissue; *c*, chimaeral shoot growing out of graft tissue. Note leaf in upper right hand corner: upper half is *N. tabacum* and lower half is GGLL. Magnification: *a*, ×0.38; *b*, ×1.5; *c*, ×0.2.

bacteria are required for tumour initiation. Since it has not been possible to demonstrate the presence of foreign DNA in sterile bacterial tumour cells[13,14], the possible genetic relationship between the suspected 'tumour inducing principle' elaborated by the bacterium and the ability of bacteria-free tumour cells to induce tumour development is unclear. The fact that genetic tumour cells cannot transmit their tumour state though crown gall cells can, suggests that genetic tumour development and maintenance results from endogenous, non-transmissible gene regulation whereas crown gall could be elicited and maintained by a transmissible factor of external origin.

CARL N. McDANIEL
IAN M. SUSSEX

*Department of Biology,
Yale University,
New Haven, Connecticut 06520*

1 Kostoff, D., *Zbl. Bakt. Parasitk de*, II abt., **81**, 244–260 (1930).
2 Smith, H. H., *Progr. exp. Tumour Res.*, **15**, 138–164 (1972).
3 Ahuja, M. R., *Molec. gen. Gent.*, **103**, 176–184 (1968).
4 Näf, U., *Growth*, **22**, 167–180 (1958).
5 Aaron-Da Cunha, M. I., *C. r. hebd. Séanc. Acad. Sci., Paris*, **268**, 318–321 (1969).
6 Meins, Jr., F., *Differentiation*, **1**, 21–25 (1973).
7 White, P. R., *Cancer Res.*, **4**, 791–794 (1944).
8 White, P. R., and Braun, A. C., *Cancer Res.*, **2**, 597–617 (1942).
9 Sussex, I. M., Clutter, M. E., and Goldsmith, M. H. M., *Am. J. Bot.*, **59**, 797–804 (1972).
10 Schaeffer, G. W., and Smith, H. H., *Pl. Physiol.*, **38**, 291–297 (1963).
11 Braun, A. G. (ed.), *Progr. exp. Tumour Res.* **15** (1972).
12 Schaeffer, G. W., *Nature*, **196**, 1326–1327 (1962).
13 Chilton, M., *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3672–3676 (1974).
14 Drlica, K. A., and Kado, C. I., *Proc. natn. Acad. Sci, U.S.A.*, **71**, 3677–3681 (1974)
15 Linsmaier, E. M., and Skoog, F., *Physiol. Pl.*, **18**, 100–127 (1965).

Target cell for oncogenic action of polycythaemia-inducing Friend virus

ONCORNAVIRUSES carry information for two distinct functions: virus multiplication and expression of oncogenicity[1,2]. The polycythaemia-inducing Friend virus (FVP) (as the Rauscher leukemia virus) infects and replicates in many types of cells cultured *in vitro* but as yet, no system for both infection *in vitro* and subsequent expression of oncongenic function has been described[3,4]. Morphological transformation of unidentified target cells occurs *in vivo* after infection of susceptible mice by FVP[5]. Transformation is characterised by the rapid and massive appearance of hyperbasophilic cells, so called Friend cells, only in the spleen red pulp, about 30–40 h after virus inoculation. Thereafter, an erythrocytic maturation starts and proceeds without influence of endogenous or exogenous erythropoietin, the physiological humoral factor for normal erythropoietic differentiation. The number of hyperbasophilic Friend cells and erythroblasts increases; these cells invade the organism and the animal dies 1–2 months after infection.

Oncogenic potencies *in vivo* could result from the need for a precise cell type or cell function to enable the expression of the oncogene[6]. Haematopoietic cells are required for the initiation of Friend disease[7]. Different authors have concluded that the target cells (that is those cells which become hyperbasophilic and multiply after virus infection) for viral oncogenic action *in vivo* were, variously, the multipotent stem cells of the haematopoietic system[8], or a myelopoietic cell[9]. Others claimed that the target cells were probably the erythropoietin responsive cells or closely related precursors[5,6].

From these indirect results, the answers to at least three questions remain unclear. First, is the multipotent stem cell of the haematopoietic system, the so-called colony forming unit, a target cell for oncogenic action of the virus? Second, is the target cell a precursor of the erythrocytic line only? Third, do the transformed hyperbasophilic cells differentiate only along the erythrocytic line?

Here we present results which, in our opinion, give direct answers to these questions, by using a method which could be easily adapted for other leukaemia viruses.

We took advantage of the well known property of the haematopoietic stem cells; they give rise, in the spleen of irradiated syngeneic recipients, to macroscopic colonies constituted of differentiated haematopoietic cells[10]. Nine days after the graft, most of the colonies contain only a single haematopoietic cell line either erythroid, neutrophilic granuloid, megakaryocytic or eosinophilic granuloid in a decreasing order of frequency[11]. Polycythaemia induced by intraperitoneal administration of packed syngeneic red cells into mice stopped the production of endogenous erythropoietin and resulted in the suppression of the erythroid spleen colonies[12]. Such suppressed erythroid colonies remain in the spleen as small undifferentiated colonies and do not convert to another cell type[13]. Three or four days after the cell graft, erythropoietin responsive cells appear in these small colonies since administration of exogenous erythropoietin results in the immediate appearance of pronormoblasts. As postulated by Lajtha, this capacity to respond to erythropoietin is achieved only after a number of cell cycles[14] (Fig. 1).

The experimental data were the following: hypertransfused, irradiated C3H/Hemg mice (Carshalton, England) were grafted with 3×10^5 syngeneic normal spleen cells (this number of cells give rise to a mean number of 10 colonies per spleen). On days 2, 4 or 6 after the graft, the mice were inoculated intravenously with 0.2 ml of highly infectious plasma (10^5 focus forming units (FFU)) from polycythaemic
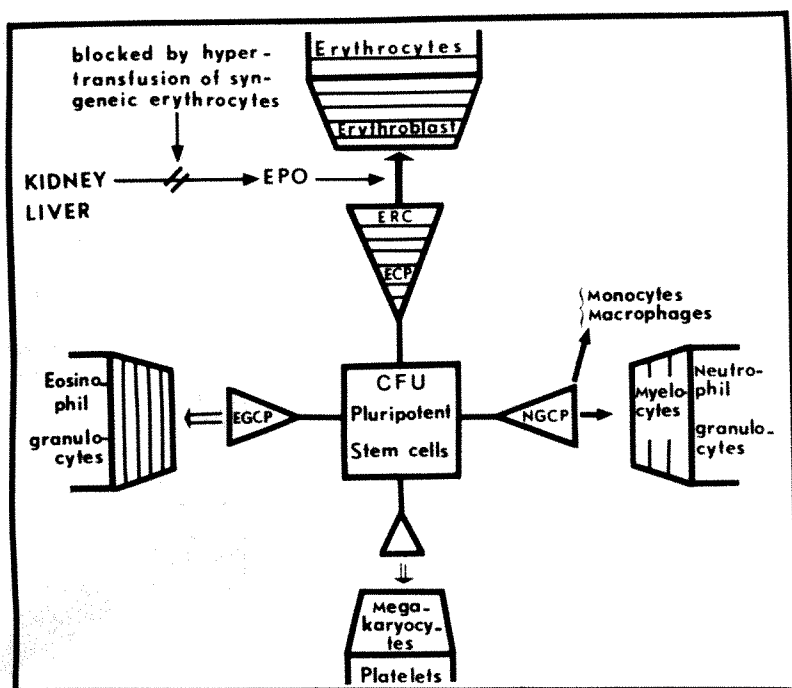
Fig. 1 Simplified schema of the murine haematopoietic system. Normal steady state[14]: The pluripotent haematopoietic stem cell compartment (CFU) is a self-maintaining population. A 'first step' differentiation event transforms the pluripotent stem cells into committed precursor cells (ECP, erythroid committed precursor; NGCP, neutrophilic granuloid committed precursor; EGCP, eosinophilic granuloid committed cells). These transit populations undergo a number of cell cycles during their maturation but without the capability for prolonged self maintenance. A 'second step' differentiation triggers the late forms of the committed cells and initiates the maturation process of the identifiable haematopoietic cell line. The mechanisms or factors which control the different haematopoietic cell line productions are as yet ill defined. The 'second step' differentiation is well understood in the case of erythropoiesis. A humoral factor erythropoietin (EPO) has been demonstrated to stimulate erythropoietin responsive cell (ERC) proliferation and recruitment, to induce ERC differentiation and to promote the erythropoietic maturation. Hypertransfused-plethoric state: Injections of washed syngeneic red blood cells suppress EPO production and result in the arrest of the erythroid maturation process. Identifiable erythroid cells disappear from haematopoietic organs but ERC remain present at any time. There is abundant evidence that the ECP is constantly supplied by an outflux from the pluripotential compartment and an intrinsic rate of ERC self-replication[15].

mice infected with FVP. Three days after virus inoculation, the spleens were removed, fixed in Helly's solution or buffered formalin with added saccharose, embedded in paraffin or methacrylate and sliced sagittally into 2–3 μm sections. Sections were stained with diorthoanisidine, an indicator of the peroxidase activity of haemoglobin, and with azur B and fuschin acid for detection of basophilic and acidophilic cells. Haemoglobin synthesis was also investigated by autoradiographic determination of ^{55}Fe incorporation. ^{55}Fe (45 μCi per mouse, specific activity 3.85 Ci g^{-1}) was injected intravenously as ferric chloride, 3 h before killing. Spleen sections mounted on glass slides were dipped in Ilford K5 emulsion and exposed for 3–4 weeks.

Spleens of mice injected with FVP on d 4 after grafting and harvested on d 7, contained as expected, the differentiated neutrophilic, megakaryocytic and eosinophilic colonies. In addition, there were small colonies of hyperbasophilic Friend cells (20–50 per section) in which baso-

philic and some polychromatophilic erythroblasts, positive to diorthoanisidine staining, were observed. The labelling of both typical hyperbasophilic Friend cells and erythroblasts in autoradiograms confirmed initiation of haemoglobin synthesis.

Spleens of mice killed 9 d after cell graft, that is 3 d after infection of FVP (day +6), contained similar but larger colonies. In these mice as well as in the former group, the distribution of the various histological types of colonies (erythroid 52%; granulocytic 28%; megakaryocytic 9%; mixed 5%; and undifferentiated 6%) was not significantly different from the distribution of colonies in spleens of non–plethoric-irradiated mice receiving the same spleen cell suspension. This indicates that practically all the presumptive erythroid colonies contained target cells for the virus. In the 9-d-old erythroid colonies obtained after injection of spleen cells into plethoric-irradiated and virus-infected hosts, the ratio of maturing erythroblasts to the large
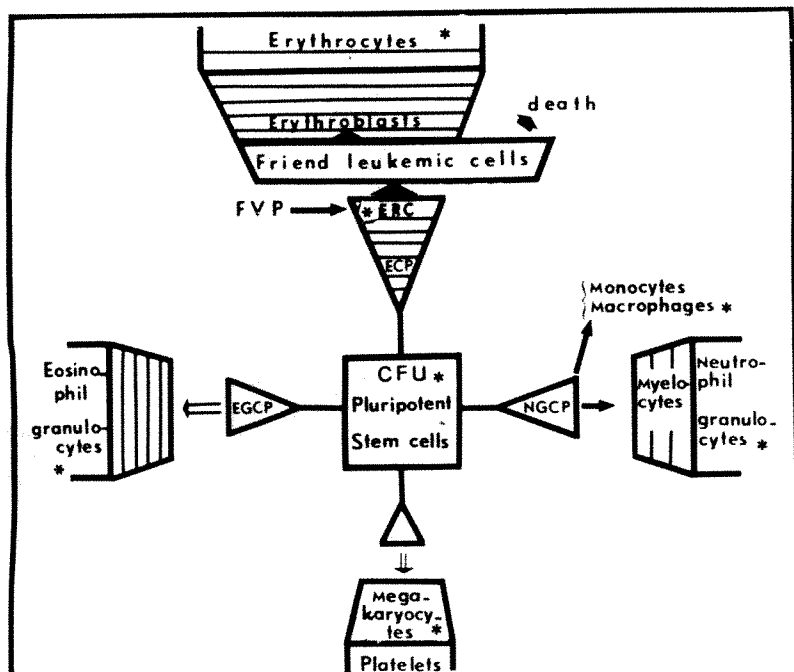
Fig. 2 Schema of the leukaemogenesis by Friend leukaemia virus. Oncogenic potency of the Friend virus (FVP) is expressed only at the 'late' erythroid committed precursor level. After viral transformation Friend leukaemic cells multiply and differentiate along the erythrocytic pathway. EPO is not required to initiate the pathological erythropoietic differentiation. As indicated by kinetic studies, Friend leukaemic cells are not self-maintaining[16] (they die or differentiate). Then the progression of the disease requires a constant recruitment, of target cells from the ECP and CFU compartments. The viral replicative ability is not restricted to the erythroblastic cells.
*The most primitive haematopoietic stem cells and the different haematopoietic cell lines are infected by the virus but their development remains practically normal.

hyperbasophilic precursor cells was smaller than in the erythroid colonies observed the spleen of irradiated normal mice, suggesting a delayed or ineffective erythropoiesis. Spleens of mice inoculated by the virus on day 2 and killed on day 5 after the cell graft, contained small clusters of 5–10 hyperbasophilic Friend cells in association with only young basophilic erythroblasts. Twenty-four hours earlier (2 d after infection) no hyperbasophilic Friends cells could be observed. Since transformation requires 30–40 h (ref. 5), it can be postulated that the target cells for the virus were present only on or after day 3 or 4. Referring to the age structure of maturation process in the erythroid committed compartment[14], this suggests that FVP needs a mature precursor cell to express its oncogenic potency.

With respect to the three initial questions, these results directly indicate that: (1) the multipotent stem cell of the haematopoietic system is not a target cell for the virus. Colony forming units (CFUs) are present in all types of colonies, and even, as shown by Trentin et al.[11], in a greater number in the granulocytic colonies. Hyperbasophilic Friend cells were, however, never observed within granuloid nor megakaryocytic colonies. (2) The viral gene which induces transformation can be expressed only in an erythroid committed cell (the erythropoietin responsive cell or a closely related precursor) and transformation is immediately followed by erythroid maturation. (3) Only the erythroid pathway of differentiation is open to hyperbasophilic Friend cells (Fig. 2).

Through use of colony formation by the multipotent haematopoietic stem cell, we have been able to examine further the early interaction between a leukaemia virus and the haematopoietic cells.

P. E. TAMBOURIN
F. WENDLING

*Unité de Physiologie Cellulaire de l'INSERM,
(Institut du Radium) Bâtiment 110,
91405 Orsay, France*

1 Huebner, R. J., and Todaro, G. J., *Proc. natn. Acad. Sci. U.S.A.*, **64**, 1087–1094 (1969).
2 Martin, G. S., *Nature*, **227**, 1021–1023 (1970).
3 Yoshikura, H., Hirokawa, Y., Yamada, M., and Sugano, H., *Jap. J. med. Sci. Biol.*, **20**, 225–236 (1967).
4 Wright, B. S., and Lasfargues, J. C., *J. natn. Cancer Inst.*, **35**, 319–327 (1965).
5 Tambourin, P., and Wendling, F., *Nature new Biol.*, **234**, 230–233 (1971).
6 Brommer, E. J. P., and Bentvelzen, P., in *Unifying Concepts of Leukemia*, 929–934 (S. Karger, Basel, 1973).
7 Chirigos, M. A., and Marsh, R. W., *Antimicrob. Agents Chemoth.*, **6**, 489–494 (1966).
8 Seidel, H. J., in *Unifying Concepts of Leukemia*, 935–942 (S. Karger, Basel, 1973).
9 Thomson, S., *Proc. Soc. exp. Biol. Med.*, **130**, 227–231 (1969).
10 Till, J. E., and McCulloch, E. A., *Radiat. Res.*, **14**, 213–222 (1961).
11 Trentin, J. J., *Regulation of Hematopoiesis*, 159–186 (Appleton–Century–Crofts, New York, 1970).
12 Schooley, J. C., *J. cell. Physiol.*, **68**, 249–262 (1966).
13 Curry, J. L., Trentin, J. J., and Wolf, N., *J. exp. Med.*, **125**, 703–720 (1967).
14 Lajtha, L. G., and Schofield, R., *Differentiation*, **2**, 313–320 (1974).
15 Reissmann, K. R., and Udupa, K. B., *Cell Tissue Kinet.*, **5**, 481–489 (1972).
16 Smadja-Joffe, F., Jasmin, C., Malaise, E. P., and Bournoutian, C., *Int. J. Cancer*, **11**, 300–313 (1973).

Characterisation of human cells transformed *in vitro* by urethane

CELLS derived from several animal species, including mouse[1,2], hamster[3] and rat[4], have been transformed *in vitro* from the normal to the malignant state by diverse chemical carcinogens. In similar conditions, however, attempts to transform human cells have usually been unsuccessful and as far as we know, such transformation has been reported only once[5]. This was a case of two cell lines treated with urethane, obtained from siblings with von Recklinghausen's disease, a familial disorder transmitted by an autosomal dominant gene, and characterised by multiple fibromas with a high predisposition to malignant

transformation *in vivo*[6]. Although morphologically altered foci of transformed cells were reported, there was the possibility that a few tumour cells in the original population had been selected for by urethane. Therefore we characterised in detail the urethane-treated and untreated cultures. We have found that human cells can indeed be chemically transformed *in vitro*.

The control cells we used were designated as NFS-1. One transformed colony (designated urethane) was derived from a single morphologically altered focus in an NFS-1 culture treated with 1.1×10^{-2} M urethane. A second transformed colony (designated urethane/FeLV) was derived from a single focus obtained in a urethane-treated NFS-1 culture preinfected with feline leukaemia virus. Since morphologically altered foci occurred in numerous urethane-treated cultures[5], whether or not infected with FeLV, there was no evidence that FeLV was acting as a cocarcinogen. We used this culture for characterisation because it is one of only two continuous cell lines obtained from various attempts to isolate and grow morphologically altered foci.

Trypsin–Giemsa banding[7] revealed (Table 1) no abnormal metaphases in the untreated NFS-1 cells, while all metaphases in the urethane cell line at passage 30 were aneuploid. Most metaphase cells contained 94 or 95 chromosomes. An abnormal marker chromosome (M_1) was found in 19 of the 20 metaphases analysed (Fig. 1). An additional marker chromosome (M_2) was found in 14 of 20 metaphases (Fig. 1). Thus the transformed cells were derived from a single parent transformed stem cell. Similar marker chromosomes were found at passage 259 (Table 1).

The urethane/FeLV line also had an aneuploid chromosomal pattern at passage 25. This pattern was different from that of the urethane cell line, since most metaphases contained between 68 and 71 chromosomes, and no metaphases had the marker M_1 or M_2 chromosome. But, four additional marker chromosomes, M_3 to M_6, not found in

Fig. 1 Marker chromosome patterns revealed by the Giemsa–trypsin banding technique: *a*, Similar marker chromosomes, M_1 and M_2, from the urethane cell line. The normal No. 7 and No. 14 human chromosomes from the same three metaphases are shown for comparison. M_1 probably arose from a deletion of the short arm of a number 7 chromosome. *b*, Representative examples of the marker chromosomes M_3–M_6 found in the FeLV/urethane cell line. M_4, M_5 and M_6 are dicentric chromosomes.
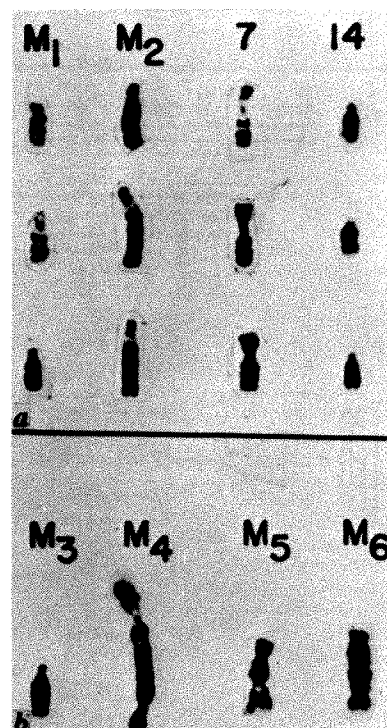
Table 1 Chromosome number and marker chromosome distribution of NFS-1 and urethane or urethane/FeLV transformed cells

Cell line	Passage no.	Chromosome no.							Number of metaphases with given markers					
		44–45	46	65–67	68–71	72–93	94–95	96–105	M_1	M_2	M_3	M_4	M_5	M_6
NFS-1	15	3	47						0	0	0	0	0	0
Urethane	30					3	14	3	19	14	0	0	0	0
	259					4	2	14	18	8	0	0	0	0
Urethane/FeLV	25			6	14				0	0	16	7	8	5

the urethane cell line were seen at various frequencies (Fig. 1). The marker chromosome M_3 was found in 16 of 20 metaphases and additional marker chromosomes M_4, M_5, and M_6 were found in 7, 8 and 5 of 20 metaphases, respectively (Table 1). This suggests clonal evolution and separate parental origin for both transformed cell lines.

Untreated NFS-1 cells formed no colonies on soft agar, whereas the established urethane cell line (Table 2) formed numerous large colonies.

We also examined the intracellular and extracellular fibrinolytic activity of the NFS-1 cells and the two urethane-treated lines because greater fibrinolytic activity is usually associated with human malignant cells than with their normal counterparts[8]. The control NFS-1 cells contained little fibrinolytic activity (that is, the level found in normal human fibroblast cultures), whereas the transformed cells showed marked fibrinolytic activity (Table 2).

We also tested the urethane cell line for its ability to produce tumours in newborn hamsters treated with anti-thymocyte serum[8]. Animals injected with 2×10^6 control NFS-1 cells were treated with antithymocyte twice weekly for the first 2 months. No tumours developed in 5 months. A similar number of cells from the urethane line, however, produced rapidly growing fibrosarcomas within 3 weeks (Table 2). When placed back in cell culture, they had the same chromosome patterns as the injected human cells, and also showed high fibrinolytic activity (data not shown). The cell line containing FeLV was not tested for tumori-genicity, since we did not wish to risk contaminating our animal facilities with FeLV.

We believe that the cultures described represent the first documented chemical malignant transformation of human cells. The possibility that a few malignant cells were selected from the NFS-1 culture can be excluded for several reasons. First, repeated attempts to develop continuous cell lines from the untreated parent cultures have failed. Second, as previously reported there was no measurable cytotoxicity nor lag in growth in the NFS-1 cells after

treatment with the doses of urethane used[5]. Third, there was no opportunity for contamination of these transformed cell lines with other human cell lines, since no long term human cell line was present in the laboratory where the work was carried out.

The strongest evidence that the two established cell lines were derived from different parent NFS-1 cells is that each line had not only a markedly different model chromosome number but also dissimilar marker chromosomes. Although the original NFS-1 cells could have two distinct malignant parent lines present before treatment with urethane, this is unlikely since previous studies have shown that human malignant tumours are most often derived from one malignant stem cell[9,10].

It is interesting that both transformed cell lines had high extracellular and intracellular fibrinolytic activity, whereas the parent cell line did not. This is consistent with studies in other species in which the parent cells contained low fibrinolytic activity, whereas chemically transformed cells had a high activity[8]. This marked difference between normal and transformed human cells may be an important criterion for the presence of human transformed cells in attempts to establish a human model system for chemical transformation. We believe that documentation of the malignant nature of these cells in culture indicates that each of the original morphologically altered colonies[5] was also malignant, but could not be developed into continuous cell lines for unknown reasons. This would be consistent with the difficulty in establishing continuous cell lines from human tumour material[11,12]. We therefore conclude that chemical transformation of human cells does occur and is frequently expressed as foci or morphologically altered cells. Tissue culture conditions, however, are usually inadequate for the establishment of long term cell lines from these transformed cells.

Table 2 Fibrinolytic activity, growth in soft agar and tumour formation of transformed and untransformed human cells

Cell line	Passage no.	Fibrinolytic activity			Colonies in soft agar per 10^4 cells plated	No. hamsters with tumours / No. hamsters injected
		Extracellular DS	HS	Intracellular U per mg protein		
NFS-1	15	1	1	1	0	0/7
Urethane	25	52	76	57.1	250	2/3
	259	58	77	69.5	342	5/7
Urethane/FeLV	25	64	68	29.9	N.D.	N.D.

Extracellular fibrinolytic activity was measured by plating 5×10^5 cells on to ^{125}I-fibrin dishes in medium containing 10% (v/v) corresponding serum (DS, dog serum; HS, human serum).

Fibrinolytic activity after 24 h, is expressed as the percentage of radioactivity released into the supernatant compared with the total radioactivity of ^{125}I-fibrin per dish[8]. Intracellular fibrinolytic activity was assayed in aliquots of cell lysates prepared in Tris-HCl buffer, pH 8.1 (0.1M) containing Triton X-100 (0.5%). The cell lysates (10 µl) were incubated for 2 h in 35 mm ^{125}I-fibrin dishes containing 2.5 ml of 1% HS in Tris-HCl, pH 8.1, and the radioactivity released was measured. The radioactivity released by 100 U of urokinase in identical conditions was taken as 100% fibrinolytic activity. One unit of activity was defined as that amount of activity which released 10% of the urokinase activity in 2 h at 37 °C. The protein content in the cell lysates was determined by the method of Lowry[11]. The methods used for the soft agar and tumorigenicity studies were similar to those previously described[8].

award from the National Institutes of Health. We thank Dr Hart Isaacs for diagnosing the tumours.

WILLIAM F. BENEDICT*
PETER A. JONES
WALTER E. LAUG

Division of Hematology–Oncology,
Department of Medicine,
Children's Hospital of Los Angeles,
and University of Southern California,
School of Medicine,
Los Angeles, California 90027

HOWARD J. IGEL
AARON E. FREEMAN

The Children's Hospital,
Buchtel Avenue and Bowery Street,
Akron, Ohio 44308

1 Chen, T. T., and Heidelberger, C., *Int. J. Cancer*, 4, 166–178 (1969).
2 Reznikoff, C. A., Brankow, D. W., and Heidelberger, C., *Cancer Res.*, 33, 3231–3238 (1973).
3 Berwald, Y., and Sachs, L., *J. natn. Cancer Inst.*, 35, 641–661 (1965).
4 Freeman, A. E., Gilden, R. V., Vernon, M. L., Wolford, R. G., Hugunin, P., and Huebner, R. J., *Proc. natn. Acad. Sci. U.S.A.*, 70, 2415–2419 (1973).
5 Igel, H. J., Freeman, A. E., Spiewak, J. E., and Kleinfeld, K. L., *In Vitro* (in the press).
6 Crowe, F. W., Schull, W. J., and Neel, J. V., *A Clinical Pathology and Genetic Study of Multiple Neurofibromatosis* (C. C. Thomas, Springfield, 1956).
7 Paul, B., Porter, I. H., and Benedict, W. F., *Humangenetik*, 18, 185–187 (1973).
8 Laug, W. E., Jones, P. A., and Benedict, W. F., *J. natn. Cancer Inst.*, 54, 173–179 (1975).
9 Atkin, N. B., and Baker, M. C., *J. natn. Cancer Inst.*, 36, 539–557 (1966).
10 Porter, I. H., Benedict, W. F., Brown, C. D., and Paul, B., *Expl molec. Path.*, 11, 340–367 (1970).
11 Freeman, A. E., and Price, P. J., in *Proc. Conf. Carcinogenesis Testing of New Drugs* (edit. by Goldberg, L.), 23–25 (Chemical Rubber Company, 1974).
12 McAllister, R. M., *et al.*, *Cancer* (in the press).

Incorporation of acetylcholinesterase into synaptic vesicles is associated with blockade of synaptic transmission

THE transmission of nerve signals across cholinergic synapses involves the presynaptic release (or secretion) of acetylcholine (ACh) which, by acting on specific sites of the postsynaptic membrane, induces changes in the postsynaptic transmembrane potential. According to the vesicle hypothesis[1], ACh is packed inside synaptic vesicles which release their contents from the nerve terminal on contact with the presynaptic membrane. Although there is abundant experimental evidence supporting the hypothesis[2], some papers question its validity[3-6]. The most important alternative hypothesis postulates that the releasable ACh is free in the cytoplasm[3-6] and that the vesicles are a secondary reservoir containing 'bound' ACh.

If the vesicle hypothesis is correct, enzymatic destruction of the ACh contained inside the vesicles should inhibit synaptic transmission. A direct way of testing the vesicle hypothesis would be to incorporate into the synaptic vesicles an enzyme that destroys the transmitter substance, and then to measure the resulting effects on transmission. Several groups[7-9] have succeeded in incorporating the enzyme horseradish peroxidase into the synaptic vesicles of various nerve terminals. In some cases[7,9], this was accomplished by stimulating the nerve terminals at a high rate for enough time to cause vesicle depletion, and then allowing the terminals to rest with the enzyme present in the bathing solution. During the rest period, vesicles containing the enzyme appeared at the terminal. Using this technique, we show here that the incorporation of exogenous acetylcholinesterase (AChE) into the synaptic vesicles of nerve terminals of the frog sartorius neuromuscular junction is associated with a marked inhibition of synaptic transmission, as would be predicted by the vesicle hypothesis.

In 16 experiments, non-curarised pairs of sartorius neuromuscular preparations, each pair taken from the same frog,
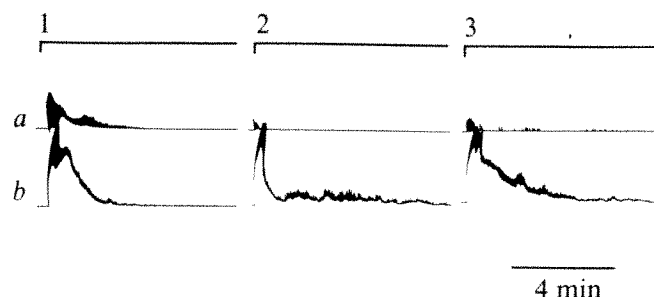
Fig. 1 Muscle contractions recorded with force transducers. *a*, Experimental; *b*, control. At the start of period 1, both preparations were stimulated simultaneously at 12 Hz. Stimulation was continued for 20 min in normal Ringer's. At the end of this period, the Ringer's solutions were exhanged for AChE–Ringer's (*a*) or for normal Ringer's (*b*), and the preparations were allowed to rest in the absence of stimulation for 45 min. Shortly before the start of period 2, both solutions were exchanged for normal Ringer's. At 2, a second cycle of stimulation identical to the first was begun. The response of E during this period was greatly diminished. Shortly before the end of period 2, the solutions were again exchanged for normal Ringer's, and the preparations were allowed to rest for an additional 45 min. At 3, a third period of stimulation was begun. Little further recovery of *a* was seen during this period.

were simultaneously stimulated through the nerves at 12 Hz for 20 min. At the end of the stimulation, the Ringer's solution bathing the experimental muscle of each pair was exchanged for Ringer's containing 0.5–1.0 mg ml⁻¹ AChE (Sigma, type V). The control muscle of each pair received either normal Ringer's, Ringer's containing 0.5–1.0 mg ml⁻¹ bovine serum albumin (Sigma), or Ringer's with paraoxon-inactivated AChE (0.5–1.0 mg ml⁻¹) dialysed against Ringer's before use. After a rest of 45 min, the bathing solutions of both experimental and control muscles were exchanged for normal Ringer's. Then the preparations were tested again for muscle contraction in response to nerve stimulation. A typical result is shown in Fig. 1. In most

Fig. 2 Endplate potentials recorded extracellularly. *a*, Experimental (top trace) and control (bottom trace) were stimulated in normal Ringer's at 20 Hz. Responses were recorded at time 0 (*a*), after 5 min of stimulation (*b*), and after 13 min of stimulation (*c*). After a total of 20 min of stimulation the bathing solutions were exchanged for AChE–Ringer's (experimental, 0.5 mg ml⁻¹) or for paraoxon-inactivated AChE–Ringer's (control, 0.5 mg ml⁻¹). The preparations then rested for 50 min. Shortly before *d*, the bathing solutions were exchanged for normal Ringer's. The response in *d* was recorded after 50 min of rest, and in *e*, after an additional 45 min of rest. The response in *f* was recorded after 9 min exposure to neostigmine methyl sulphate (0.5 mg ml⁻¹). The experimental showed no recovery of endplate potential during the later periods of stimulation, *d*, *e*, or *f*.

0.2 mV
10 ms
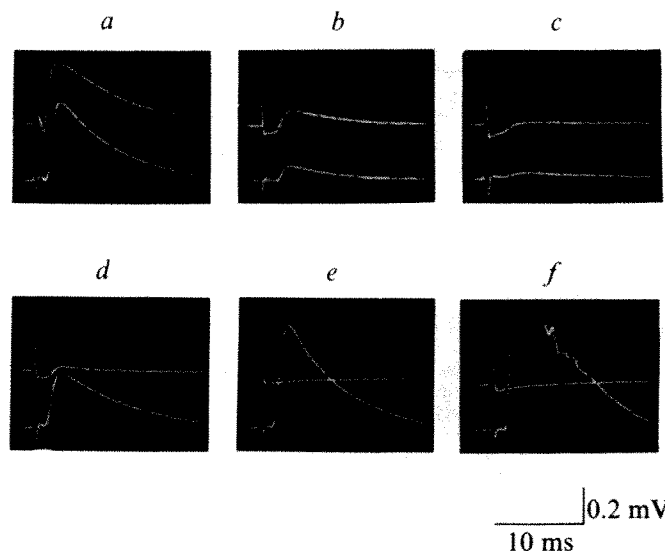
cases (13 out of 16) the experimental preparation showed an obvious diminution in contraction, whereas the control showed a significant recovery. Occasionally, it was necessary to repeat the cycle of stimulation and rest (in one case, four times) to elicit the effect. The fact that paraoxon-inactivated esterase and albumin did not inhibit transmission indicates that ACh hydrolysis was necessary.

In a different series of experiments, Fatt's method of extracellular recording of endplate potentials (e.p.p.s) was used[10]. Paired preparations were curarised (1–1.5 mg l^{-1} D-tubocurarine, Abbot) and AChE- and albumin-containing Ringer's were used following the sequence described in Fig. 2. As in non-curarised preparations, the AChE treatment (in seven of nine cases) greatly diminished or abolished the postsynaptic response to nerve stimulation, whereas the response of control preparations recovered to the pre-stimulation value. In these experiments too, the procedure in some cases had to be repeated more than once to obtain the effect. Preincubation of the preparations at 4 °C or brief stimulation during the rest period seemed to interfere with the effect.

Several attempts to reverse the AChE effect were made with both curarised and non-curarised preparations by introducing a second stimulation period (20 Hz, 20 min), in AChE-free Ringer's. Heuser and Reese[7] reported that a second stimulation of terminals already loaded with exogenous horseradish peroxidase resulted in the disappearance of enzyme-containing vesicles. In our experiments, however, no recovery of the AChE-inhibited preparations was observed.
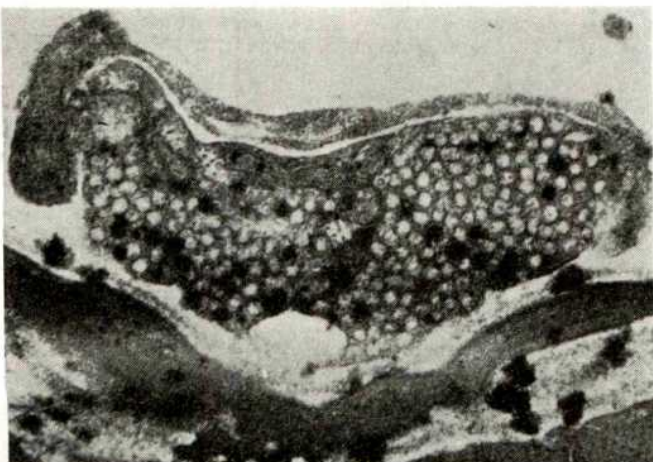
Fig. 3 Electron micrograph of nerve terminal stained for incorporated AChE ($\times 67,500$). The nerve–muscle preparation was stimulated at 20 Hz for 20 min and rested for 45 min in AChE–Ringer's. After washing several times with normal Ringer's, the preparation was fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.4, and then stained for cholinesterase activity[14], osmicated, dehydrated, and embedded in Epon. Dimethyl sulphoxide (0.1%) was included in the staining reagent. Black deposits in the synaptic vesicles indicate the presence of AChE activity. Controls which were stimulated and rested in normal Ringer's and then treated as above, had no reaction product in the vesicles. Deposits of reaction product in the synaptic cleft were also seen in controls.

The above mentioned inhibition of the postsynaptic response could be explained by hydrolysis of ACh in the synaptic cleft. There is, in fact, some evidence that AChE can enter and leave the cleft[11–13]. To test this possibility, preparations were stimulated at a slow rate (about 2 min^{-1}) in the presence of AChE (0.5–1.0 mg ml^{-1}) in the Ringer's. With this non-fatiguing stimulation no effects on muscle contraction were observed for several hours. Presumably, vesicle recycling and incorporation of exogenous AChE at this rate of stimulation occurs only very slowly. Additional evidence against the extracellular action of exogenous

AChE is the fact that in preparations where synaptic transmission was completely blocked as described above, the addition of a cholinesterase inhibitor (neostigmine methyl sulphate, K & K, 1 mg l^{-1}) did not restore the e.p.p.s (Fig. 2f).

To determine the location of the exogenous AChE in the presynaptic terminal after stimulation to fatigue and rest in the presence of the enzyme, preparations so treated were fixed, and then stained for the demonstration of cholinesterases according to Crevier and Belanger[14]. In the preparation shown in Fig. 3, 15–20% of the synaptic vesicles contain electron-dense reaction product indicative of cholinesterase activity.

These results show that the incorporation of exogenous AChE into synaptic vesicles is associated with inhibition of synaptic transmission at the frog sartorius neuromuscular junction, suggesting that the ACh available for release is contained in the synaptic vesicles. These results, however, do not rule out the possibity that there is a releasable cytoplasmic store of ACh which is in equilibrium with the intravesicular pool. This latter possibility is compatible with the results of Tauc *et al.*[6] who showed that injection of AChE into the axoplasm of an *Aplysia* cholinergic neurone resulted in inhibition of transmission.

An interesting further application of the technique described here might be the use of this or other transmitter-inactivating enzymes to identify the transmitter substance in different synapses.

ALBERTO POLITOFF
ALAN L. BLITZ

Department of Physiology,
Boston University School of Medicine,
80 East Concord Street,
Boston, Massachusetts 02118

STEVEN ROSE

Department of Neurosciences,
Albert Einstein College of Medicine,
1300 Morris Park Avenue,
Bronx, New York 10461

1 del Castillo, J., and Katz, B., *J. Physiol., Lond.*, **128**, 396–411 (1955).
2 Katz, B., *Nerve, Muscle and Synapse* (McGraw-Hill, New York, 1966).
3 Dunant, Y., Gautron, J., Israel, M. Lesbats, B., and Manaranche, R., *J. Neurochem.*, **19**, 1987–2002 (1972).
4 Molenaar, P. C., Polak, R. L., and Nickolson, V. J., *J. Neurochem.*, **21**, 667–678 (1973).
5 Richter, J. A., and Marchbanks, R. M., *J. Neurochem.*, **18**, 705–712 (1971).
6 Tauc, L., Hoffmann, A., Tsuji, S., Hinzen, D. H., and Faille, L., *Nature*, **250**, 496–498 (1974).
7 Heuser, J., and Reese, T., *J. Cell Biol.*, **57**, 315–344 (1973).
8 Holtzman, E., and Peterson, E. R., *J. Cell Biol.*, **40**, 863–869 (1969).
9 Ceccarelli, B., Hurlbut, W. P., and Mauro, A., *J. Cell Biol.*, **57**, 499–524 (1973).
10 Fatt, P., *J. Physiol., Lond.*, **114**, 408–422 (1950).
11 Betz, W., and Sakmann, B., *J. Physiol., Lond.*, **230**, 673–688 (1973).
12 Hall, Z. W., and Kelly, R. B., *Nature new Biol.*, **232**, 62–63 (1971).
13 Kreutzberg, G. W., and Kaiya, H., *Histochemistry*, **42**, 233–237 (1974).
14 Crevier, M., and Belanger, L. F., *Science*, **122**, 256–257 (1955).

Differences between detergent-extracted acetylcholine receptor and "cholinergic proteolipid"

SNAKE venom α-toxins[1] in combination with reversible[2,3] or alkylating[4,5] cholinergic ligands give a selective labelling of the cholinergic (nicotinic) receptor protein (AChR)[6–8]. Since this protein is membrane-bound and therefore expected to possess hydrophobic properties, two different methods have been used for its dissolution and purification: (1) chloroform–methanol extraction[8] and (2) non-denaturing detergent extraction in aqueous media[2,3]. The former solubilises the so-called "cholinergic proteolipid" which, still in organic media, binds charged cholinergic ligands[9]. The latter procedure has made possible the purification of the

AChR by affinity chromatography and the study of the binding of the above-mentioned ligands in physiological media (for reviews see refs 6–8). Furthermore, the serum of rabbits immunised against the purified AChR[10–12] blocks *in vivo* the physiological response to cholinergic agonists[10,11], thereby confirming that the detergent-extracted AChR is indeed involved in the permeability change caused by acetylcholine. On the other hand, the physiological importance of the "cholinergic proteolipid" remains undetermined, though the challenge to the actual purification[13] has recently been refuted[14]. It is therefore the aim of the present work to compare the "cholinergic proteolipid" and the detergent-extracted AChR from the same sources: the electric organs of *Electrophorus electricus* and *Torpedo marmorata* using the selective labels α-toxin[15] and the covalent affinity reagent 4-(N-maleimido)-phenyltrimethylammonium iodide[4] in combination with immunodiffusion tests[11].

Frozen electric tissue from *Electrophorus electricus* was thawed, homogenised in water, lyophilised and extracted with chloroform–methanol (2 : 1 v/v) as described by La Torre *et al.*[16]. The crude organic extract was partitioned with one fifth its volume of water, and the proteolipids of the organic phase precipitated with four volumes of diethyl ether at −20 °C. After standing overnight, the precipitate was redissolved in chloroform–methanol (2 : 1 v/v), concentrated *in vacuo*, and chromatographed through the organophilic dextran gel Sephadex LH 20 using chloroform–methanol mixtures[16]. The proteolipid fraction III (the so-called "receptor proteolipid")[9,16] was isolated for further study. In other series of experiments, fresh and lyophilised electric tissue were extracted with chloroform–methanol at various temperatures (25 °C, −50 °C, −80 °C) according to ref. 17. After evaporation of the organic solvent at +25 °C or −25 °C, the samples were re-extracted by adding Triton X-100 in *Electrophorus* Ringer's solution (160 mM NaCl, 5 mM KCl, 2mM $MgCl_2$, 2 mM $CaCl_2$, 2.5 mM Na phosphate buffer pH 7) to a final 1% detergent concentration and stirred for 1 h at room temperature. The detergent extracted fractions were analysed for their binding activity with ^3H-α-*Naja nigricollis* toxin (15 Ci mmol^{-1}) (ref. 15) and for their ability to react with rabbit antibodies raised against purified acetylcholine receptor from *Electrophorus* (anti-AChR), using the Ouchterlony immunodiffusion test[11] (Fig. 1). The Triton X-100 extracted AChR was purified from *Electrophorus* membrane fragments by affinity chromatography[18].

Fig. 1 Ouchterlony double diffusion in 1% agarose gel and 0.025 M Veronal buffer pH 8.2. 1, Triton X–100 extract of proteolipid fraction III (ref. 16); 2, 3, AChR purified by affinity chromatography in the presence of Triton X–100 (ref. 18); 4, 5, 6, 7, Anti-AChR sera raised in four different rabbits. The presence of the Triton X-100-extract of proteolipid fraction III in hole No. 1 does not induce any deviation of the precipitation lines between AChR and anti-AChR: no cross reaction can be seen between AChR and the proteolipid fraction III.
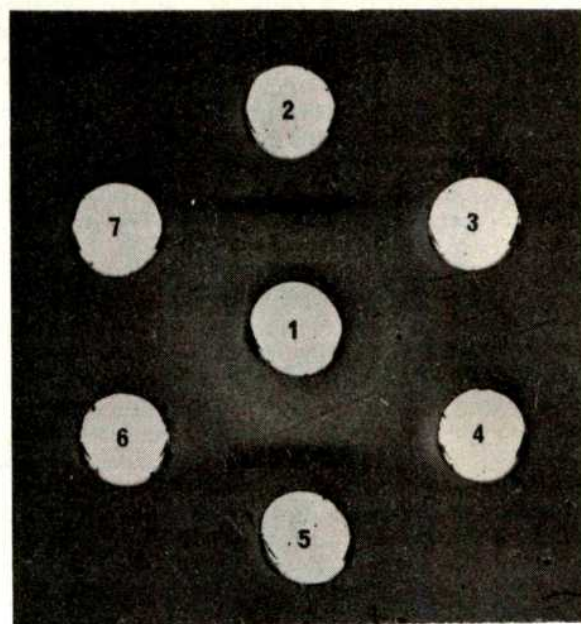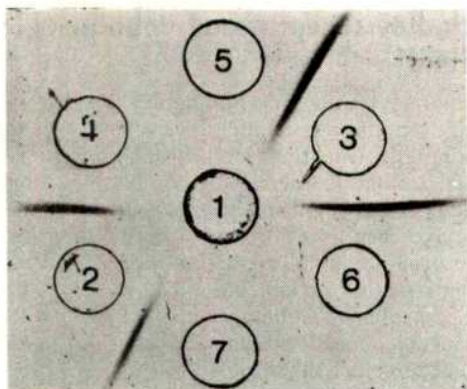
Fig. 2 Ouchterlony double diffusion in 1% agarose gel and 0.023 M Veronal buffer pH 8.2. 1, Anti-proteolipid fraction III sera, raised in two different rabbits; 2, 5, Proteolipid fraction III in 1% Triton X–100; 3, 4, 6, 7, AChR purified by affinity chromatography in 1% Triton X–100. Precipitation lines are seen between hole 1 and holes 2 and 5. The presence of AChR in holes 3, 4, 6 and 7 induces no deviation of the precipitation lines: no cross reaction occurs between AChR and rabbit anti-proteolipid fraction III sera.

Rabbit antisera were also raised against proteolipid peak III from *Electrophorus electricus* electric tissue. Three rabbits each received 500 μg proteolipid peak III in 0.5 ml Freund's adjuvant; the dose was repeated after one week and after a further 2 weeks. The ability of the rabbit antibodies to react with proteolipid peak III and with purified AChR was tested using the Ouchterlony immunodiffusion test[11] (Fig. 2).

Regardless of the temperature of extraction[17] and subsequent concentration, the proteolipid protein transferred to 1% Triton X-100 or emulsified in water, before or after chromatography on Sephadex LH 20, does not show binding of ^3H-α-toxin nor does it react against anti-AChR antisera. Similarly, no cross reaction between the AChR[18] and the organic extract or the purified "cholinergic proteolipid" of La Torre *et al.*[16] was observed in these immunodiffusion tests.

In another series of experiments 25 μg of the AChR protein purified by affinity chromatography[18] (2,400 nmol ^3H-α-toxin bound per g of protein) was incubated overnight with Cl_3CH–CH_3OH (2 : 1 v/v) at 0 °C, 4 °C and 25 °C. After evaporation of the organic solvent with N_2, the residual aqueous samples were assayed for α-toxin binding and immunoprecipitation. In all cases, the ability to bind the cholinergic ligand and to react against the anti-AChR antisera were lost. The immunological and the toxin binding assays on water-solubilised proteolipids might be negative because of a possible partial denaturation of the so-called "receptor proteolipid" during transfer to aqueous solvents, although the detergent-solubilised proteolipid retains its antigenicity to rabbit anti-proteolipid peak III serum as seen in Fig. 2. The AChR was therefore covalently labelled, before its extraction with organic solvents, with 4-(N-maleimido)-phenyltri-^3H-methylammonium (^3H-MPTA), by a procedure similar to that used by Karlin and Cowburn[5] with 4-(N-maleimido)-benzyltri-^3H-methylammonium (^3H-MBTA). This allowed the AChR to be followed

Table 1 ³H-MPTA labelling of AChR-rich membrane fragments from *T. marmorata* (800 nmol ³H-α-toxin bound per g of protein)

	³H-MPTA
In the absence of α-toxin	2,400 c.p.m. μg⁻¹ protein
After preincubation with 30 μg ml⁻¹ of unlabelled α-toxin	8 c.p.m. μg⁻¹ protein
CM extraction of labelled membrane fragments	
(1) From aqueous phase (15 μg protein)	
CM extract	50 c.p.m.
Aqueous phase	34,600 c.p.m.
(2) From lyophilised membranes (48 μg protein)	
CM extract	2,500 c.p.m.
Solid residue	110,000 c.p.m.

Chloroform—methanol (2:1 v/v) (CM) extraction of labelled membrane fragments : the receptor-rich membrane fragments from the electric organ of *Torpedo marmorata* were prepared according to ref. 19. They were then labelled with ³H-MPTA following the procedure of Karlin[5]. The receptor-rich membrane fragments (800 nmol α-toxin sites per g of protein) were incubated for 10 min in the presence of 1 mM dithiothreitol in buffer I (0.1 M NaCl, 1 mM EDTA, 0.01M Tris-HCl, pH 8). This reduction was stopped by adding 0.5 M Na Phosphate buffer pH 6.7 and cooling to 0 °C. After centrifugation at 100,000g for 90 min, the pellet was resuspended in buffer II (0.15 M NaCl, 1 mM EDTA, 0.01 M Na phosphate buffer pH 7.0). An aliquot was incubated for 90 min at 20 °C with an excess of α-toxin from *Naja nigricollis*, the rest being incubated without α-toxin at the same temperature. The two samples were then run in parallel. They were labelled with ³H-MPTA (about 2 Ci mmol⁻¹) for 2 min at 25 °C. The labelling was stopped with 2-mercaptoethanol. The membrane fragments were then washed by threefold centrifugation followed by resuspension of the pellet in buffer III (0.15 M NaCl, 1 mM EDTA, 0.01 M sodium phosphate buffer, pH 7.0). (1) The labelled membrane fragments were extracted with CM. The extract was partitioned with H₂O and the "theoretical upper phase"[16] was prepared with Cl₃CH : CH₃OH—H₂O (3:48:47 by volume) and separated by centrifugation (3,000g, 10 min). This procedure was done three times and the radioactivity was measured in the combined upper phases and in the washed CM extract. (2) The membrane fragments were first lyophilised and then extracted with CM, and filtered. The radioactivity was measured in the residue and the CM extract.

even in a denatured state. Table 1 shows the results of the affinity labelling with ³H-MPTA of the AChR-rich membrane fragments from *Torpedo marmorata* prepared according to ref. 19.

The native membrane fragments bound 800 nmol of ³H-α-toxin per g of protein; after reduction by dithiothreitol, 2,400 c.p.m. of ³H-MPTA (about 2 Ci mmol⁻¹) were covalently attached per μg of protein. Preincubation of the membrane fragments with 30 μg ml⁻¹ α-toxin before affinity alkylation reduces by 99.7% the amount of ³H-MPTA retained by the membrane fragments confirming that, under these conditions, the labelling of ³H-MPTA is specific for the AChR site.

Samples of the ³H-MPTA labelled membrane fragments were extracted with chloroform—methanol (2 : 1 v/v) and the crude extract extensively washed with H₂O (Table 1). No radioactivity was recovered in the organic phase. All the radioactivity was recovered in the combined aqueous phases and in a precipitate which formed at the water–chloroform–methanol interface. When the chloroform–methanol (2 : 1 v/v) extraction was performed on lyophilised tissue, as described by La Torre *et al.*[16], only 1.7% of the radioactivity was found in the organic extract, the rest being recovered in the residue.

The experiments using affinity alkylation of the membrane-bound AChR from *Torpedo marmorata*, in coincidence with those carried out on the material extracted from *Electrophorus electricus*, indicate that in agreement with Karlin[7] the AChR subunit(s) carrying the site(s) for binding α-toxin or MPTA and recognised by the anti-AChR antisera[10-12] are not extracted with chloroform–methanol (2:1 v/v). The organic-extracted "cholinergic proteolipids"[9,16,17] from the same sources are therefore different

from the detergent-extracted AChR in all these respects.

F. J. BARRANTES

Max-Planck Institut für biophysikalische Chemie, 3400 Göttingen-Nikolausberg, West Germany

J. P. CHANGEUX

Neurobiologie Moléculaire, Institut Pasteur, Paris, France

G. G. LUNT

Biochemistry Department, University of Bath, UK

A. SOBEL

Neurobiologie Moléculaire, Institut Pasteur, Paris, France

1 Lee, C. Y., *Clinical Toxicology*, 3, 457–472 (1970).
2 Changeux, J. P., Kasai, M., and Lee, C. Y., *Proc. natn. Acad. Sci. U.S.A.*, 67, 1241–1247 (1970).
3 Miledi, R., Molinoff, P., and Potter, L. T., *Nature*, 229, 554–557 (1971).
4 Karlin, A., and Winnik, M., *Proc. natn. Acad. Sci. U.S.A.*, 60, 668–674 (1968).
5 Karlin, A., and Cowburn, D., *Proc. natn. Acad. Sci. U.S.A.*, 70, 3636–3640 (1973).
6 Cohen, J. B., and Changeux, J. P., *A. Rev. Pharmac.*, 15, 83–103 (1975).
7 Karlin, A., *Life Sci.*, 14, 1385–1415 (1974).
8 Rang, H. P., *Q. Rev. Biophys.*, 7, 283–399 (1975).
9 De Robertis, E., *Science*, 171, 963–971 (1971).
10 Patrick, J., and Lindstrom, J., *Science*, 180, 871–872 (1973).
11 Sugiyama, H., Benda, P., Meunier, J. C., and Changeux, J. P., *FEBS Lett.*, 35, 124–128 (1973).
12 Heilbronn, E., and Mattson, C., *J. Neurochem.*, 22, 315–317 (1974).
13 Levinson, S. R., and Keynes, R. D., *Biochim. biophys. Acta*, 288, 241–247 (1972).
14 Donellan, J. F., and Cattell, K. J., *Biochem. Soc. Trans.*, 3, 106–109 (1975).
15 Menez, A., Morgat, J. L., Fromageot, P., Ronseray, A. M., Boquet, P., and Changeux, J. P., *FEBS Lett.*, 17, 333–335 (1971).
16 La Torre, J. L., Lunt, G. S., and de Robertis, E., *Proc. natn. Acad. Sci. U.S.A.*, 65, 716–720 (1970).
17 Izumi, F., and Freed, S., *FEBS Lett.*, 41, 151–155 (1974).
18 Meunier, J. C., Sealock, R., Olsen, R., and Changeux, J. P., *Eur. J. Biochem.*, 45, 371–394 (1974).
19 Cohen, J. B., Weber, M., Huchet, M., and Changeux, J. P., *FEBS Lett.*, 26, 43–47 (1972).

Possible mechanism for action of prolactin on mammary cell sodium transport

PROLACTIN, like other polypeptide and glycoprotein hormones from the pituitary gland, exerts its effects on target cells through interaction with receptors on the plasma membrane[1-4]. Unlike several other pituitary hormones, this does not occur through activation of adenyl cyclase[5], and the mechanism of action of prolactin must therefore lie elsewhere.

In this study we have investigated a possible mechanism which is derived from the earlier (in evolution), osmoregulatory function of prolactin. In estuarine fishes, prolactin maintains internal osmolarity by regulating Na⁺ diffusion across the gills, and by controlling the activity of Na⁺/K⁺-activated ATPase, the active monovalent ion pump, in gills and kidney[6]. Prolactin may therefore act similarly on mammary alveolar cells, which are of epithelial origin, through the regulation of monovalent cation transport.

Measurement of sodium²² transport in response to prolactin, growth hormone and albumin was carried out using slices of lactating mammary tissue incubated with ²²Na⁺ as described in Table 1. The results of these experiments show a highly significant decrease in tissue ²²Na⁺ after addition of prolactin, and a less significant decrease after growth hormone. The growth hormone preparation had less than 0.5 IU per mg prolactin potency, but it is possible that this was sufficient to cause the decrease.

In further experiments, incubation of mammary tissue slices in the presence of 5 × 10⁻⁵ M ouabain resulted in a 10% in-

crease in tissue $^{22}Na^+$ content. Incubation with ouabain plus prolactin abolished the decrease in $^{22}Na^+$ observed after prolactin alone, and resulted in tissue $^{22}Na^+$ content similar to incubations with ouabain alone.

In these experiments, tissue $^{22}Na^+$ content after incubation would be caused by $^{22}Na^+$ entry by diffusion, opposed by the activity of the Na^+ pump (Na^+/K^+-activated ATPase) in extruding intracellular Na^+. Ouabain selectively inhibits this Na^+ pump[8], and was used in these experiments to demonstrate whether the reduced $^{22}Na^+$ content of the tissue caused by prolactin occurred as a result of lowered Na^+ entry or accelerated Na^+ extrusion. As ouabain abolished the prolactin effect, the data suggest that the action of prolactin is on the Na^+ pump to increase Na^+ transport out of the cell.

Monovalent ion concentrations in lactating mammary tissue were measured after incubation in the presence or absence of prolactin. With rabbit tissue (Table 2), and with guinea pig tissue (I.R.F. and J.M.R., unpublished), significantly lower tissue Na^+ concentrations resulted from incubation with prolactin. Tissue K^+ concentrations did not change significantly, but tissue Cl^- was significantly decreased after incubation with prolactin. Because of the permeability of the apical membranes of the cells to K^+ and the high concentration gradient to be expected between intra- and extracellular K^+ in this system, any changes in cell K^+ transport may well be masked.

The observed decrease in tissue Na^+ when prolactin is present in the incubation indicates that prolactin directly stimulates the transport of Na^+ out of the cell, presumably by increasing the activity of Na^+/K^+-activated ATPase. To confirm this, we are at present investigating the influence of prolactin on isolated mammary membrane fragments having Na^+/K^+-activated ATPase activity. Note that cytological studies have located both the Na^+/K^+-activated ATPase and the prolactin receptors on the same restricted region of the alveolar cell membrane[2,10]. The studies of Cowie[11] on milk composition in the rabbit provide supporting evidence for the effect of prolactin on monovalent ion transport, as he showed a rise in milk Na^+ and Cl^- in late lactation, which could be reversed by prolactin. As the ionic composition of milk reflects alveolar intracellular ionic composition[12], it is likely that monovalent cation concentration in the milk is regulated by ion transport at the basal membrane of the cell, and thus would be responsive to prolactin.

It seems likely therefore that an early response of mammary alveolar cells to prolactin is the activation of the Na^+ pump leading to a decreased intracellular Na^+ concentration, and a

Table 1 Effect of prolactin, growth hormone or albumin on $^{22}Na^+$ content of mammary gland slices

Treatment	Concentration (μg protein per mg tissue)	$^{22}Na^+$ content of 0.1 g slices (c.p.m.±s.e.m.) (3 experiments, 6 replicates in each)
Control	—	376.2 ± 16.5
Prolactin	0.5	326.8 ± 13.3†
Growth hormone	0.5	342.6 ± 13.8*
Albumin	0.5	381.1 ± 18.5

$*P \simeq 0.05$; $†P < 0.01$.

Lactating mammary alveolar tissue was obtained from rabbits 10–20 d *post partum*, killed by cervical dislocation with minimum disturbance. For 3 d before killing, bromocriptin (CB154, gift from Professor E. Flukiger and Dr H. Friedle, Sandoz, Basle) was administered daily at 1 mg kg^{-1} subcutaneously, to inhibit prolactin secretion[7]. The mammary alveolar tissue was cooled, chopped into 0.7 mm slices (McIwain chopper), and washed in Tyrode's solution (*p*H 7.4) at 0 °C before use. Prolactin (NIH P/S9), growth hormone (NIH B 17GH gifts from the National Institutes of Health) and bovine serum albumin (Commonwealth Serum Laboratories, Melbourne) dissolved in Tyrode's solution were used as described. To measure $^{22}Na^+$ transport, 0.1 g tissue slices were incubated in 2 ml Tyrode's solution containing 1% rabbit serum at 37 °C for 2 h in an oscillating (200 cycles min^{-1}) water bath, in the presence of 0.1 μCi $^{22}Na^+$. To this incubation medium were added 20 μg prolactin, growth hormone or albumin. After incubation the supernatant was decanted, the slices washed at 0 °C for 15 min in Tyrode's solution, blotted and $^{22}Na^+$ content determined in a well-type scintillation counter.

Table 2 Monovalent ion concentrations in slices of lactating mammary alveolar tissue from rabbits after incubation with increasing concentrations of proclactin

Prolactin concentration ($M \times 10^{-8}$)	Monovalent ion concentration (meq kg^{-1} tissue; mean ± s.e.m.)		
	Na^+	K^+	Cl^-
0.00	114.1 ± 2.1	23.7 ± 0.9	90.2 ± 2.7
4.35	107.9 ± 2.2*	25.3 ± 1.2	78.3 ± 4.2†
8.70	104.8 ± 3.4†	22.9 ± 1.0	82.5 ± 2.7*
17.40	105.0 ± 2.2†	25.1 ± 1.1	84.0 ± 3.0

$*P < 0.05$; $†P < 0.01$.

Lactating rabbits were used on three separate occasions for these experiments and the data combined. Each rabbit was treated with bromocriptin as described in Table 1. Each experiment comprised 20 samples of 500 mg 0.7 mm slices of mammary tissue, incubated in Krebs bicarbonate buffer, *p*H 7.4, for 1 h with continuous bubbling of 95% O_2–5% CO_2. Prolactin was added to the incubation medium in three sets of five samples in each experiment at the concentrations shown, which correspond to 0.05, 0.1 and 0.2 μg per mg tissue. After incubation, the tissue was homogenised with an overhead homogeniser (Ultra-Turrax, Type TP1810 Janke and Kunkel K.G., Staufen i. Breisgau) in 4 ml deionised water. Monovalent ion content was measured using an Atomic Absorption Spectrophotometer (AA100, Techtron, Melbourne, Australia) and the chloride content determined by titration[9].

marked change in the intracellular Na^+/K^+ ratio. These changes may act as an intracellular messenger in the biosynthetic events resulting from prolactin stimulation, as alterations in monovalent cation concentrations have been shown to stimulate nuclear activity in insect salivary glands in a hormone-like response[13]. Investigations of mammary alveolar cell responses to changes in monovalent cation concentrations may thus shed light on the mechanism of action of prolactin.

We thank the Nuffield Foundation for support, and Mr T. Buckley and Mrs K. Tadros for assistance.

IAN R. FALCONER
J. M. ROWE

Department of Biochemistry and Nutrition,
University of New England, Armidale,
New South Wales, Australia 2351

1 Turkington, R. W., *Biochem. biophys. Res. Commun.*, **41**, 1362–1367 (1970).
2 Birkinshaw, M., and Falconer, I. R., *J. Endocr.*, **55**, 323–334 (1972).
3 Shiu, R. P. C., and Friesen, H. G., *J. biol. Chem.*, **249**, 7902–7911 (1974).
4 Shiu, R. P. C., and Friesen, H. G., *Biochem. J.*, **140**, 301–311 (1974).
5 Turkington, R. W., in *Lactogenic Hormones* (edit. by Wolstenholme, G. E. W., and Knight, J.), 111–127 (Churchill-Livingstone, Edinburgh and London, 1972).
6 Ensor, D. M., and Ball, J. N., *Fedn Proc.*, **31**, 1615–1623 (1972).
7 Hart, I. C., *J. Endocr.*, **57**, 179 (1973).
8 Skou, J. C., *Physiol. Rev.*, **45**, 596–617 (1965).
9 Cotlove, E., Tranthan, H. V., and Bowman, R. L., *J. Lab. clin. Med.*, **31**, 461–468 (1958).
10 Kinura, T., J., *Japan Obstet. Gynec. Soc.*, **21**, 301–308 (1969).
11 Cowie, A. T., *J. Endocr.*, **44**, 437–450 (1969).
12 Linzell, J. M., and Peaker, J. L., *Physiol. Rev*, **51**, 564–597 (1971).
13 Kroeger, H., in *Endocrine Genetics* (edit. by Spickett, S. G.), *Endocr. Soc. Symp.*, **15**, 55–66 (Cambridge University Press, Cambridge, 1967).

Internal regulation of iron absorption

McCANCE and Widdowson[1] concluded that body iron content is regulated by variation in the amount absorbed and not by variation in excretion, and many workers have since attempted to define the factors which relate iron absorption to the needs of the body. Although there is a large body of data on the intracellular mechanisms of absorption[2] this has not advanced our understanding of the physiology of regulation.

There are three main theories describing the regulation of iron absorption. The first proposes that this control is exercised mainly by the gut mucosa. The epithelial cells of the small intestine incorporate iron from the plasma during their development, and it has been suggested that the amount taken up by these cells reflects the iron status of the body and that this in turn conditions them to allow the passage of more or less iron[3]. Within the cell this

control may be related to the cell's ability to synthesise ferritin[3] (which may retain iron and so prevent the transfer of unwanted iron to the plasma), to the degree of saturation of a hypothetical carrier protein[3,4] or to the iron requirements of the cell for haem synthesis[5]. Changes in the rate of plasma iron turnover have, however, been shown to affect iron absorption and it has been thought that this, rather than changes in plasma iron concentration, may be the main determinant of the iron status of the developing epithelial cell[6].

The second, related, theory proposes that control is a function of the different characteristics of the two iron-binding sites of the transferrin molecule in the plasma[7]. The number of molecules with two iron atoms will, it is assumed, reflect the level of body iron stores and determine the concentration of iron in the mucosal cells. This may then regulate the transfer from the mucosa to the plasma as in the first theory. It is suggested, however, that one transferrin iron-binding site is preferentially relieved of iron by the red cell precursors of the marrow and is also particularly avid for intestinal iron. This superimposes a second controlling factor on iron absorption whereby it may be related to the demands of erythropoiesis as well as to the level of the iron stores[7].

The third theory involves a humoral factor which is secreted either in direct or inverse proportion to body iron requirements and which signals the gut to increase or decrease iron absorption. Conrad[8] has commented on a number of experiments exploring this hypothesis.

There is no direct experimental evidence to support either the transferrin or the humoral control theories. The mucosal mechanisms which have been proposed do not explain all the observed phenomena and indeed recent observations have cast doubt on some of the earlier conclusions. These were based on measurements of the iron content of the whole gut wall and seemed to show an inverse relationship between mucosal iron and iron absorption[6,9]. Direct measurement of the iron content of epithelial cell homogenates has now shown that there is no direct relationship between epithelial cell iron and absorption[10-12]. Moreover, iron-deficient epithelial cells have a capacity for ferritin synthesis equal to that of normal cells[13]. The iron or ferritin content of the mucosal cell does not seem to be the controlling factor in iron absorption. The basic question remains — how is iron absorption induced to respond to the rate of erythropoiesis and the level of iron stores? We suggest that this regulation may best be explained by a consideration of internal iron exchange.

Iron circulating in the plasma bound to transferrin exchanges with iron in all cells of the body. An exchangeable iron pool may be defined as iron available for binding by circulating transferrin irrespective of its site or chemical form. Its size will depend not only on cellular iron concentration but also on the transferrin-binding sites in different tissues. The probability of an iron atom from a given tissue being picked up by a free receptor site on a transferrin molecule is proportional to the ratio of exchangeable iron in that tissue to the exchangeable iron in the whole body. The number of iron atoms picked up in this way per unit time is equal to the number cleared from the plasma. This is reflected in the experimentally measured plasma iron turnover. Iron transfer from the intestinal epithelium to the plasma is therefore proportional to

$$\frac{\text{Intestinal exchangeable iron}}{\text{Total exchangeable iron}} \times \text{Plasma iron turnover}$$

In general the total exchangeable iron in the body is directly related to the level of storage iron and our hypothesis explains why low iron stores are associated with increased iron absorption and high stores with reduced

absorption. It predicts that this relationship will hold not only in patients with iron deficiency or iron overload but also in normal subjects; this has been observed[14,15]. This hypothesis also shows how an increase in iron absorption may follow an increase in plasma iron turnover as a result of erythroid overactivity. It also explains why this is not always found. When increased erythropoiesis is associated with haemolysis, increased haemoglobin catabolism results in an increased amount of iron in the reticuloendothelial system. This increases the total exchangeable iron and will counterbalance the effect of plasma iron turnover on iron absorption, and thus a stable haemolytic anaemia may be associated with normal iron absorption[16].

In conclusion, the present hypothesis offers a simple rational explanation of the relationship between several endogenous factors which can influence iron absorption, and provides a framework for further investigation.

I. CAVILL
M. WORWOOD
A. JACOBS

Department of Haematology,
Welsh National School of Medicine,
Cardiff, UK

1 McCance, R. A., and Widdowson, E. M., Lancet, ii, 680–684 (1937).
2 Turnbull, A., in Iron in Biochemistry and Medicine (edit. by Jacobs, A., and Worwood, M.), 369–403 (Academic, London and New York, 1974).
3 Crosby, W. H., Blood, 22, 441–449 (1963).
4 Pinkerton, P. H., Ann. Int. Med., 70, 401–408 (1969).
5 Jacobs, A., in Clinics in Haematology, 2, no. 2, (edit. by Callender, S. T.), 323–337 (Saunders, London 1973).
6 Weintraub, L. R., Conrad, M. E., and Crosby, W. H., Blood, 24, 19–24 (1964).
7 Fletcher, J., and Huehns, E. R., Nature, 218, 1211–1214 (1968).
8 Conrad, M. E., Gastroenterology, 57, 225–228 (1969).
9 Conrad, M. E., Weintraub, L. R., and Crosby, W. H., J. clin. Invest., 43, 963–974 (1964).
10 Balcerzak, S. P., and Greenberger, N. J., Nature, 220, 270–271 (1968).
11 Richmond, V. S., Worwood, M., and Jacobs, A., Br. J. Haemat., 14, 605–614 (1972).
12 Mattii, R., Mielke, C. H., Levine, P. H., and Crosby, W. H., Blood, 42, 959–966 (1973).
13 Brittin, G. M., and Raval, D., J. Lab. clin. Med., 75, 811–817 (1970).
14 Cook, J. D., Lipschitz, D. A., Miles, L. E. M., and Finch, C. A., Am. J. clin. Nutrit., 27, 681–687 (1974).
15 Walters, G. O., Jacobs, A., Worwood, M., Trevett, D., and Thomson, W., Gut, 16, 188–192 (1975).
16 Crosby, W. H., in Regulation of Hematopoiesis, 1 (edit. by Gordon, A. S.), 519–537 (Appleton–Century–Crofts, New York, 1970).

Appearance of hypoxanthine guanine phosphoribosyltransferase activity as a consequence of mycoplasma contamination

MYCOPLASMAS are common contaminants of cells in culture and are often responsible for profound alterations in the metabolism of infected cells[1,2]. Such alterations include chromosomal aberrations[3,4] and changes in host cell enzyme activities[5]. We have reported that amniotic fluid cell cultures contaminated with mycoplasmas revealed a significant increase in chromosomal aberrations over those seen in uninfected cultures[6]. This finding demonstrated that the presence of these organisms in amniotic fluid cell lines poses a potential hazard to prenatal chromosome diagnosis.

In addition to chromosomal disorders many inherited metabolic disorders can now be diagnosed prenatally, including biochemical defects involving diminished or complete absence of enzyme activities[7]. The studies reported here were designed to determine whether cells lacking a specific enzyme activity may acquire significant levels of activity as a consequence of mycoplasma infection. The cell line chosen for these studies was D98/AH-2, a human cell line which lacks measurable hypoxanthine guanine phosphoribosyltransferase (HGPRT) activity[8]. HeLa cells were used as a positive control for human HGPRT activity.

D98/AH-2 and HeLa cells were grown in Eagle's basal medium supplemented with 10% calf serum, 28 mM HEPES buffer and 100 U ml^{-1} penicillin and subcultured

twice weekly. Actively dividing D98/AH-2 cells were infected with 1×10^6 colony forming units (CFU) of *Acholeplasma laidlawii* or *Mycoplasma hyorhinis*, two common contaminants of cell cultures[1,2]. The infected cells were assayed for HGPRT activity 7 d and 28 d after infection. The HGPRT activities of the two mycoplasma strains were assayed separately.

Representative HGPRT activities are shown in Table 1. Pellets of both *A. laidlawii* and *M. hyorhinis* contained high levels of HGPRT activity. D98/AH-2 cells, as expected, had no measurable activity. Significant levels of HGPRT activity were however found in the infected cultures. In particular, cells infected with *M. hyorhinis* had levels of activity similar to that of the HeLa control. The levels of HGPRT activity probably represent a conservative estimate of the actual activity since 5–40% of inosinic acid was apparently converted to inosine and was not included in our calculated activities. This was probably due primarily to mycoplasmal 5'-nucleotidase activity[10], although D98/AH-2 5'-nucleotidase activity may have been a contributory factor. It is of interest to note that cells infected with *M. hyorhinis* had much higher levels of enzyme activity than did cells infected with *A. laidlawii*, whereas both mycoplasma strains alone have comparable activity.

An attempt was made to correlate activity with a degree of contamination by measuring the CFUs of mycoplasmas in the cell pellets. This provides a relatively crude estimate of the number of viable mycoplasmas[11], but it permits the inference that the low HGPRT activities in cells infected with *A. laidlawii* were due to lower mycoplasma : cell ratios than those cultures infected with *M. hyorhinis*. This is further supported by the fact that no inhibition of mycoplasmal HGPRT activity was seen when D98/AH-2 cells were mixed with high numbers of *A. laidlawii* just before the HGPRT assay (Fig. 1), indicating that D98/AH-2 cell extract was not inhibitory to the acholeplasma HGPRT. In a separate series of experiments hypoxanthine was added to cell cultures at the time of infection in an attempt to induce higher levels of mycoplasmal HGPRT activity. As shown in Table 1 no significant increase was noted in D98/AH-2 culture infected with *M. hyorhinis*. The apparent increase in HGPRT activity in cells infected with

A. laidlawii in the presence of hypoxanthine may merely represent the inaccuracy of the quantitative assay of viable mycoplasmas associated with the cells.

Electrophoretic analysis of mycoplasmal and cell extracts was done to compare the mobilities of the mycoplasmal and human HGPRT enzymes. Slab gel electrophoresis of infected cells showed bands of activity only for those infected with *M. hyorhinis* (Fig. 1). Distinct bands due to *A. laidlawii* activity were seen only when these mycoplasmas and uninfected cells were mixed together just before extraction. Both mycoplasmal HGPRT activities migrate to a position distinct from that of human HGPRT.

We wish to draw notice to the extra band arrowed in the HeLa cell profile in Fig. 1. These cells were obtained from a laboratory subsequently shown to possess mycoplasma contaminated cell lines (E.J.S. and J.T., unpublished observations). Unfortunately this particular cell line was lost before routine mycoplasma testing was performed. All subsequent electrophoretic analyses of HeLa HGPRT activity using cells known to be free of mycoplasma did not, however, reveal this band.

Our results clearly indicate that the presence of mycoplasmas as contaminants of cell cultures can lead to altered enzyme patterns. This finding is of considerable importance since it suggests that errors in prenatal diagnosis may occur if amniotic fluid cell cultures are contaminated with mycoplasmas. Of particular relevance is the prenatal diagnosis of the Lesch–Nyhan syndrome in which HGPRT activity is diminished[12]. We have also demonstrated significant levels of hexosaminidase A activity in mycoplasmas (E.L.S., J. Tallman, and E.J.S., unpublished observations); the enzyme which is used for pre- and postnatal diagnosis of Tay-Sachs disease[13]. Mycoplasmas also express adenine phosphoribosyltransferase activity at levels higher than those observed in cultured human cells when calculated on a total protein basis (J.A.T. and E.J.S., unpublished observations).

The degree of mycoplasma contamination reached in our deliberately infected cultures is comparable with levels encountered in accidentally contaminated cell lines. Because of the high frequency of mycoplasma contamination found in tissue culture facilities we believe it is

Table 1 HGPRT activities of *A. laidlawii* and *M. hyorhinis* in cell cultures

Sample	HGPRT activity (c.p.m. in inosinic acid/µg protein/min of assay)	Approximate no. of viable mycoplasmas/0.1 ml packed cell volume (± 0.5 log$_{10}$)
A. laidlawii	27.46	3.0×10^9
M. hyorhinis	17.45	1.1×10^9
D98/AH-2 cells	No activity	Negative
D98/AH-2 cells infected with *M. hyorhinis*	18.27	1.9×10^8
D98/AH-2 cells infected with *M. hyorhinis* in the presence of 10^{-4} M hypoxanthine	5.58	8.0×10^7
D98/AH-2 cells infected with *A. laidlawii*	No activity	2.0×10^7
D98/AH-2 cells infected with *A. laidlawii* in the presence of 10^{-4} M hypoxanthine	2.87	2.0×10^7
HeLa	17.75	Negative

Mycoplasmas were grown in broth for 2–4 d at 37 °C, then collected by centrifugation at 10,000 r.p.m. for 20 min, washed twice with phosphate buffered saline (PBS) and the pellets stored at -37 °C until the time of assay. Cell cultures were collected by trypsinisation, washed twice with PBS, pelleted and stored at -70 °C. In one series of experiments 10^{-4} M hypoxanthine was added to the growth medium at the time of infection in an attempt to induce higher levels of mycoplasmal HGPRT activity. Extracts of mycoplasma and cell pellets were prepared by sonication. The assay was started by the addition of the sample extract to 100 µl of reaction mixture containing 0.25 µCi ^{14}C-hypoxanthine (specific activity 5.37 Ci mM^{-1}); magnesium chloride 5 mM; 5-phosphoribosyl-1-pyrophosphate 1 mM; Tris buffer (pH 7.4) 55 mM; and bovine serum albumin 150 µg. The reaction was carried out at 37 °C and samples removed at 0,10 and 30 min. The reaction in each case was stopped by the addition of formic acid (to 0.66 M) and immersion in a dry ice–methanol bath. Hypoxanthine, inosine and inosine monophosphate were separated by spotting 5-µl samples on Eastman Chromogram Cellulose TLC sheets using non-radioactive markers for identification and 5% Na$_2$HPO$_4$ as a solvent. Spots were visualised with ultraviolet light, cut out and counted by scintillation spectrometry.

3 Paton, G. R., Jacobs, J. P., and Perkins, F. T., *Nature*, **207**, 43–45 (1965).
4 Stanbridge, E., Onen, M., Perkins, F. T., and Hayflick, L., *Expl Cell Res.*, **57**, 397–410 (1969).
5 Rose, N. R., Milisauskas, V., and Kite, J. H., Jr, *Proc. Soc. exp. Biol. Med.*, **140**, 390–394 (1972).
6 Schneider, E. L., *et al.*, *Science*, **184**, 477–480 (1974).
7 Nadler, H. L., *Adv. Human Genet.*, **3**, 1–38 (1972).
8 Szybalski, W., Szybalska, E. H., and Ragni, G., *Natn. Cancer Inst. Monogr.*, **7**, 75–89 (1962).
9 Tischfield, J. A., Bernhard, H. P., and Ruddle, F. H., *Analyt. Biochem.*, **53**, 545–554 (1973).
10 Razin, S., *J. Microbiol.*, **28**, 243–250 (1962).
11 Brunner, H., James, W. D., Horswood, R. L., and Chanock, R. M., *J. Immun.*, **108**, 1491–1498 (1972).
12 DeMars, R., Santo, G., Felix, J. S., and Benke, P., *Science*, **164**, 1303–1305 (1969).
13 Okada, S., and O'Brien, J. S., *Science*, **165**, 698–700 (1969).
14 Harris, H., *Birth Defects*, **9**, 132–137 (1973).
15 Bakay, B., Croce, C. M., Koprowski, H., and Nyhan, W. L., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1998–2002 (1973).
16 Tischfield, J. A., and Ruddle, F. H., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 45–49 (1974).

Fig. 1 Separation of mycoplasmal and human HGPRT activities. Electrophoretic analysis was done according to the method of Tischfield *et al.*[9]. The technique involves slab gel electrophoresis followed by lanthanum precipitation of the charged, labelled product and autoradiography of the dried gel. *a*, *M. hyorhinis*; *b* and *c*, D98/AH-2 infected with *M. hyorhinis*; *d*, *A. laidlawii*; *e*, D98/AH-2 and *A. laidlawii* mixed just before electrophoresis; *f*, HeLa control for human HGPRT. Note the extra band (arrowed) which may be due to accidental mycoplasma contamination (see text).

imperative that prenatal diagnostic centres should institute routine screening of their amniotic fluid cultures for mycoplasmas. Since cultured skin fibroblasts are also used for the postnatal biochemical diagnosis of many inherited metabolic disorders, mycoplasma contamination could interfere with these determinations as well. If further studies show that mycoplasma contaminants of cell cultures have enzyme activities which migrate distinctly from human enzymes by gel electrophoresis, then this procedure could be used to distinguish between their respective activities.

These results should also serve as a warning to investigators involved in somatic cell genetics, where contaminating mycoplasmal enzyme activities may be confused with isoenzyme expression[14] and 'reactivation' of enzyme activities after somatic cell hybridisation[15]. A further potential hazard to this area of cell biology is the known capacity of mycoplasmas to induce chromosomal aberrations, including translocations[3,4,6], which could lead to problems of assigning gene activities to specific chromosomes[16].

ERIC J. STANBRIDGE

Department of Medical Microbiology,
Stanford University School of Medicine,
Stanford, California 94305

JAY A. TISCHFIELD

Department of Biology,
Case Western Reserve University,
Cleveland, Ohio 44106

EDWARD L. SCHNEIDER

Laboratory of Cellular and Comparative Physiology,
NIA, NIH, DHEW and Baltimore City Hospitals,
Baltimore, Maryland 21224

1 Stanbridge, E., *Bact. Rev.*, **35**, 206–221 (1971).
2 Fogh, J., Holmgren, N. B., and Ludovici, P. P., *In Vitro*, **7**, 26–41 (1971).

IMP dehydrogenase, an enzyme linked with proliferation and malignancy

PREVIOUS studies conducted in this laboratory demonstrated that in a spectrum of liver tumours with different growth rates there is an imbalance in the activities of key enzymes and competing pathways of carbohydrate, pyrimidine, DNA and ornithine metabolism[1–4]. Recent investigations on purine metabolism showed that in the hepatomas there was an increased capacity in the *de novo* pathway of biosynthesis of inosine 5′-monophosphate (IMP), as reflected in the increased activity of glutamine PRPP amidotransferase (EC 2.4.2.14) and a decrease in IMP catabolism[5,6]. These observations directed our attention to the metabolic fate of IMP because this purine nucleotide is at a strategic position in purine metabolism (Fig. 1). Control at such branching points is exerted primarily by modification of the activity or of the rate of synthesis of the first enzymes of the divergent pathways. In this instance, in normal conditions, a balance is maintained by both positive and negative feedback effects. Thus ATP is required for GMP biosynthesis and GTP for AMP biosynthesis; conversely, GMP and AMP each inhibit their own production[7]. In normal or malignant proliferation, or in response to a hormone stimulus, changes in control frequently involve reprogramming of gene expression. The theoretical framework and predictive value of generalisations about such biochemical changes (termed the molecular correlation concept) have been elaborated[1,3].

Briefly, in tumours, three classes of behaviour of enzymes and pathways were distinguished. Enzymes or pathways of the first group show a positive or negative correlation with tumour growth rate; these alterations seem to indicate a linking with the degree of expression of malignancy. Parameters of the second group are consistently increased or decreased in all tumours, regardless of growth rate, and thus seem to be linked with the malignant transformation *per se*. The third group includes parameters which show apparently random increases or decreases[1]. For a fuller understanding of the control of the guanine nucleotide pathway, therefore, it became necessary to study the first enzyme of this pathway, IMP dehydrogenase (IMP: NAD oxidoreductase, EC 1.2.1.14) in conditions of normal and neoplastic growth. This enzyme has been examined in several mammalian tissues[8–18], including mouse sarcoma cells[12] and human placenta[14]. Comparative studies in homologous normal and malignant tissues have not, however, been reported.

IMP dehydrogenase cannot be measured accurately in crude tissue homogenates, because of interfering activities. The extraction technique and optical assay described in the legend to Fig. 2 were developed, from earlier methods[10,12], to give optimal activity with liver and hepatoma preparations. In the conditions described, rates were linear for at least 30 min, and proportional to enzyme concentration up to a level of 0.0015 IU ml^{-1}. No measurable nucleotidase activity was present. Normal and tumour-bearing
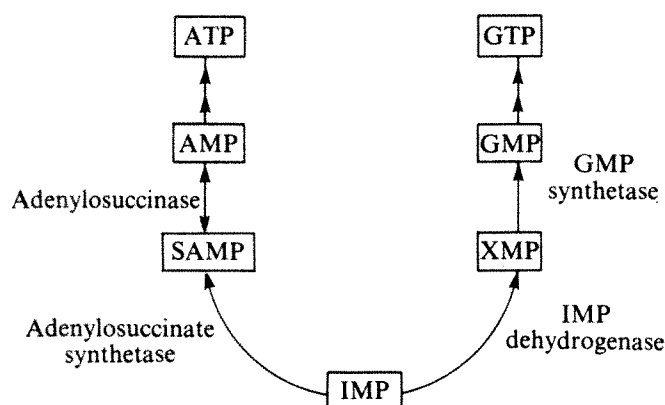
Fig. 1 Simplified schematic metabolic map of the two principal competing routes for utilisation of inosine 5'-monophosphate. IMP, inosine 5'-monophosphate; SAMP, adenylosuccinate; XMP, xanthosine 5'-monophosphate; AMP and ATP, adenosine 5'-mono- and triphosphates; GMP and GTP, guanosine 5'-mono- and triphosphates, respectively.

rats were maintained as described previously and the preparation of regenerating liver, studies on differentiating animals, killing of rats and excision of livers and neoplasms were as reported elsewhere[1-3]. The growth rates of the various hepatoma lines ranged from 2 weeks to 11.5 months, measured by the time required for the tumours to reach a diameter of 1.5 cm. The IMP dehydrogenase activity and protein content in 11 transplantable hepatomas and in livers of control normal rats are compared in Fig. 2. In liver, the mean activity was 2,140 pmol substrate converted per h per mg of protein present in the 100,000g supernatant (coefficient of variation 15%). One gram (wet weight) of liver contained 88 mg of soluble protein. IMP dehydrogenase activity was elevated in all the hepatomas examined; even in the slowest growing tumours, with intervals of over a year between successive transplants, at least a 2.5-fold increase was seen. Moreover, the enzyme activity correlated with growth rate, and the increase, relative to normal liver, reached 13-fold in the most rapidly proliferating hepatomas. All increases were statistically significant at the 0.1% level, and the coefficient of correlation of IMP dehydrogenase activity with the exponential growth rate constant was 0.945 ($P<0.001$). The enzyme activity in renal tumour MK3 was increased threefold over the value observed in normal control kidney cortex.

It was important to determine whether the IMP dehydrogenase of hepatomas had different properties from the liver enzyme. For this purpose the rapidly growing hepatoma 3924A was chosen. The pH profiles of the liver and hepatoma enzymes were identical, with optima at pH 8.1. Both enzymes were inhibited by excess NAD$^+$ at concentrations above 0.25 mM, the inhibition reaching 50% at 0.62 mM. Both enzymes were also subject to product inhibition by XMP (competitive with IMP and non-competitive with NAD$^+$) and by NADH (non-competitive with both IMP and NAD$^+$). Liver and hepatoma dehydrogenases were inhibited equally by NADPH (50% inhibition, under usual assay conditions, at 0.14 mM) and by GMP (competitive with IMP, K$_i$ 96 mM). These patterns, together with initial velocity data, suggested that the kinetic mechanism of liver and hepatoma IMP dehydrogenases was identical with that of the sarcoma 180 and human placenta enzymes[12,14]. Within the limits of experimental error, kinetic parameters were identical for the liver and hepatoma enzymes, and were as follows (the terminology is that of Cleland[17]): K_a for IMP, 11.6±2.5 μM; K_{ia} for IMP, 34.6±4.6 μM; K_b for NAD$^+$, 24.3±3.8 μM; K_i for XMP, 25.9±3.1 μM; K_i (slope) for NADH, 360±66 μM; K_i (intercept) for NADH, 260±10 μM. Thus, the hepatoma

IMP dehydrogenase has properties similar to those of the normal liver enzyme.

To study the behaviour of IMP dehydrogenase in proliferating, non-malignant, adult hepatic tissue, we used the technique of partial hepatectomy[18]. Figure 3 shows that in the regenerating liver IMP dehydrogenase activity started to rise 6 h after operation, reached a peak of about five times the normal activity by 24 h, and then declined rather slowly. It is noteworthy how early the activity started to increase; most of the key enzymes of nucleic acid biosynthesis only start to rise after 12 h[2,18]. Another valuable system for the study of control of hepatic enzymes is the neonatal liver, which provides a population of differentiating, proliferating liver cells. On a basis of enzyme activity per unit of wet weight, the activity of IMP dehydrogenase in livers of 1-d-old rats is nearly four times the adult level, slowly declining thereafter with increasing age. But the average size of liver cells in newborn rats is less than 40% of the adult size. When our results were expressed on the basis of IMP dehydrogenase activity per cell (cell counts being determined by visual counting of orcein-stained nuclei in homogenates[1]) the per-cell activity in neonatal liver was only slightly (25%) higher than the adult value.

To evaluate this enzyme in the context of cell proliferation and function in the whole animal we measured IMP dehydrogenase in a wide variety of rat tissues. The highest activities were found in thymus, spleen, bone marrow and testis, which were, respectively, 10.5, 8.6, 3.3 and 2.1 times the liver specific activity. Successively lower activities were observed in brain, lung, intestinal mucosa, renal cortex, adipose tissue, red blood cells, and skeletal and cardiac muscle.

Fig. 2 IMP dehydrogenase activity in rat hepatomas. 10% (w/v) homogenates were prepared in 0.25 M sucrose, buffered to pH 7.4 with Tris-chloride. 100,000g supernatants were fractionated with ammonium sulphate, and the fraction precipitating between 30% and 40% saturation contained all the IMP dehydrogenase activity. This precipitate was resuspended and dialysed against 2 × 200 volumes of assay buffer (0.1 M potassium phosphate, pH 7.4 plus EDTA, 1 mM and mercaptoethanol, 0.5 mM). Samples were assayed at 290 nm, in the same buffer, at 37 °C in a Gilford 2400-S recording spectrophotometer. The concentration of NAD$^+$ in the assay was 0.2 mM and that of IMP was 0.17 mM. The protein concentration of the 100,000g supernates was determined by the Biuret reaction. The data are means ±s.e. of five or more animals in each group and results are plotted as percentages of corresponding control liver values.
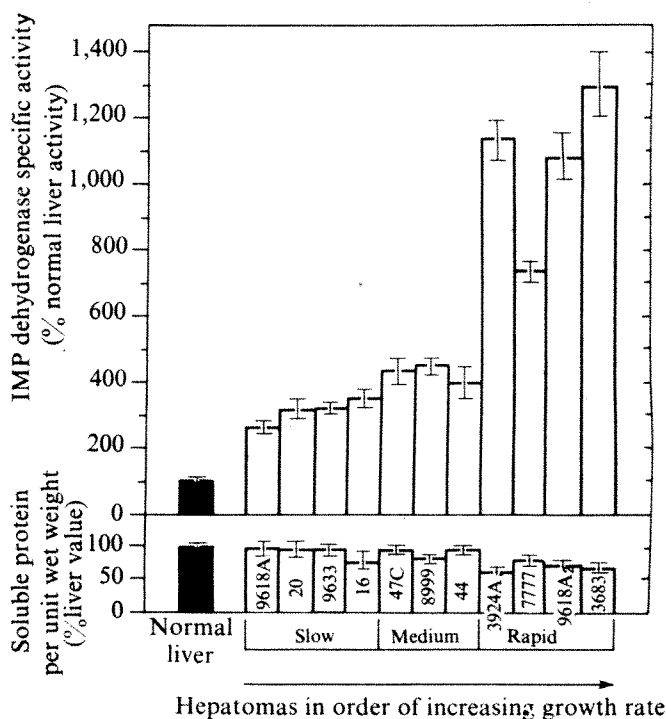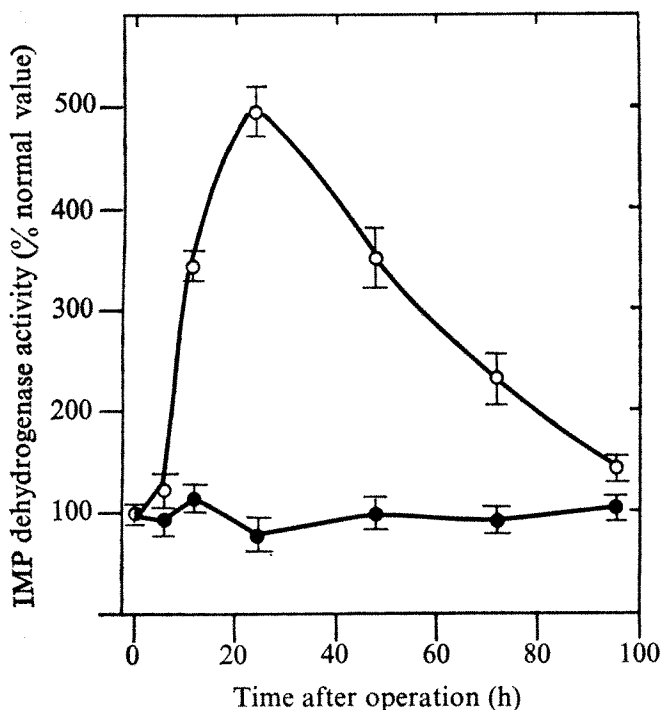
Fig. 3 IMP dehydrogenase in rat liver following partial hepatectomy (○) and sham operation (●). Enzyme was prepared and assayed as described in Fig. 2.

From this study a number of conclusions may be drawn. First, a relationship of IMP dehydrogenase activity with cell proliferation is evident. This correlation is most clearly apparent in the spectrum of transplantable hepatomas of different growth rates, and is further emphasised by the high activity found in regenerating liver, and in thymus and spleen. The correlation, however, is not absolute, as the lower activities in neonatal liver and in intestinal mucosa demonstrate. Second, the low absolute activity of this enzyme, even in the most rapidly dividing tissues, suggests that it may be one of the key rate-controlling enzymes in nucleic acid biosynthesis. The activity found in regenerating liver, for example, would be sufficient to allow the liver RNA and DNA content to be doubled in about 18 h. In fact, the doubling time for regenerating liver is about 4 d; it seems that under these conditions, and also in the fastest-growing tumours, IMP dehydrogenase must operate at around 20% of its maximum velocity. Last, the regulation of the synthesis of this enzyme is undoubtedly changed in malignancy. Even the slowest growing hepatomas with mass-doubling times of more than 40 d, have a 2.5 to 3.5-fold elevation compared with normal liver, and the rapid hepatomas, with growth rates comparable with that of regenerating liver[1,3], have, on average, almost twice as much IMP dehydrogenase activity as is observed at the peak following partial hepatectomy. Moreover, in hepatomas, the enzyme activity elevation is permanent.

The ubiquitous increase of IMP dehydrogenase in hepatomas indicates the linking of this enzyme alteration with the neoplastic transformation. Superimposed on this reprogramming of gene expression is the positive correlation of this enzyme activity with the rate of growth of the liver neoplasms, indicating a linking of this enzymatic imbalance also with the expression of the degrees of malignancy.

This work was supported by grants from the US Public Health Service.

ROBERT C. JACKSON
GEORGE WEBER

*Laboratory for Experimental Oncology and
Department of Pharmacology,
Indiana University School of Medicine,
Indianapolis, Indiana 46202*

HAROLD P. MORRIS

*Department of Biochemistry,
Howard University College of Medicine,
Washington DC 20001*

1 Weber, G., in *The Molecular Biology of Cancer* (edit. by Busch, H.), 487–521 (Academic, New York, 1974).
2 Weber, G., Queener, S. F., and Ferdinandus, J. A., *Adv. Enzyme Regul.*, 9, 63–95 (1971).
3 Weber, G., *Adv. Enzyme Regul.*, 11, 79–102 (1973).
4 Williams, J. C., Weber, G., and Morris, H. P., *Nature*, 253, 567–569 (1975).
5 Katunuma, N., and Weber, G., *FEBS Lett.*, 49, 53–56 (1974).
6 Weber, G., Prajda, N., and Williams, J. C., *Adv. Enzyme Regul.*, 13, 3–25 (1975).
7 Hartman, S. C., in *Metabolic Pathways*, third ed., 4 (edit. by Greenberg, D. M.), 1–69 (Academic, New York, 1970).
8 Abrams, R., and Bentley, M., *Archs Biochem.*, 56, 184–195 (1955).
9 Atkinson, M. R., Morton, R. K., and Murray, A. W., *Biochem. J.*, 89, 167–172 (1963).
10 Al-Mudhaffar, S., and Ackerman, C. J., *Endocrinology*, 82, 912–918 (1968).
11 Pehlke, D. M., McDonald, J. A., Holmes, E. W., and Kelley, W. N., *J. clin. Invest.*, 51, 1398–1404 (1972).
12 Anderson, J. A., and Sartorelli, A. C., *J. biol. Chem.*, 243, 4762–4768 (1968).
13 McFall, E., and Magasanik, B., *J. biol. Chem.*, 235, 2103–2108 (1969).
14 Holmes, E. W., Pehlke, D. M., and Kelley, W. N., *Biochim. biophys. Acta*, 364, 209–217 (1974).
15 Saccoccia, P. A., and Miech, R. P., *Molec. Pharmac.*, 5, 26–29 (1969).
16 Sweeney, M. J., Hoffman, D. H., and Esterman, M. A., *Cancer Res.*, 32, 1803–1809 (1972).
17 Cleland, W. W., *Biochim. biophys. Acta*, 67, 104–137 (1963).
18 Bucher, N. L. R., and Malt, R. A., *Regeneration of Liver and Kidney* (Little, Brown, Boston, 1971).

Efficiency of light conversion by the blue-green alga *Anacystis nidulans*

THE conversion efficiency of light energy into chemical energy, measured by production of organic matter, can be high with unicellular organisms. As photosynthesis becomes light saturated at relatively low light intensities, such high efficiencies are not usually obtained with high intensities of incident light. The saturation level, however, increases with increasing temperature. Blue-green algae grow well at high temperatures[1-3], suggesting that they may be efficient producers of organic matter. As the protein content of algae in general is high, growth of these organisms could have important implications for food production, especially in tropical areas, where both light intensity and temperature are favourable. We have therefore investigated some parameters influencing yield of production of *Anacystis nidulans* at high light intensities.

High growth rates require an adequate supply of carbon dioxide for cell production. As a result of a high ratio of outer surface area chlorophyll content, favourable for CO_2 diffusion, unicellular organisms can profit much more from enhanced CO_2 availability than leaves of higher plants.

Using a medium according to Kratz and Myers[4] we found that an increase in A_{680} of 42% (the *in vivo* chlorophyll maximum) resulted from 6 h illumination with incandescent lamps (50 W m^{-2} photosynthetically active light), if the cells were flushed with air enriched with 5% CO_2. The absorbance increase for a culture flushed with plain air was only 10% of that of cells flushed with enriched air, indicating that CO_2 was a limiting factor in these conditions. In the latter culture the phycocyanin–chlorophyll ratio, measured by A_{625} and A_{680}, decreased from a value of 1.4 to 1.8, found with rapidly growing cells, to about 0.8 (Fig. 1). A marked increase in growth rate and in phycocyanin–chlorophyll ratio resulted if these cultures were switched from air to CO_2-enriched air. Such an increase also followed when the rate of flushing with air was tenfold increased and the bubble size decreased. If the culture was flushed with plain air but 2% sodium bicarbonate was added to the culture medium and the pH readjusted to 7.5, growth proceeded at a rate similar to that of cells flushed by CO_2-enriched air. After 1.5 d, however, the pH of this culture had risen to 11, phycocyanin was bleached and the cells eventually died.

An intensity of 50 W m^{-2} from incandescent lamps was not sufficient for maximal growth with cells flushed with CO_2-enriched air, indicating that photosynthesis was not

saturated. If the intensity was doubled, the rate of absorption increase also doubled (temperature 40 °C, flushing rate 8 l h^{-1}).

After 4 d continuous illumination with 50 W m^{-2}, growth stopped. Removal of the cells by centrifugation and renewed inoculation with fresh cells in the same phase of growth as at the start of the experiment did not result in resumed growth. Investigation of the ultraviolet absorption spectrum of the supernatant suggested that at least the nitrogen source was depleted. Removal of the cells, renewed inoculation and addition of 50% fresh growth medium resulted in a vigorous growth for 18 h. After that time the cells turned yellow and growth stopped. The absorption spectrum of the yellow cells and absorption difference spectra with normal cells indicated that phycocyanin disappears, chlorophyll concentration decreases and that of a carotenoid increases markedly (Fig. 1). Thin-layer chromatography of acetone extracts from the cells showed that the same acetone-soluble pigments are present in both types of cell, but the yellow ones contain, besides less chlorophyll, an appreciably greater content of a xanthophyll. Ultraviolet absorption spectra of the supernatant indicated the excretion of various polypeptides[5], which may act as growth inhibitors and modifiers of the absorption spectrum.

Anacystis receiving light from fluorescent tubes (Philips TL 33, also equivalent to 50 W m^{-2}) grow at a similar rate to cells illuminated with incandescent lamps in the same conditions of CO_2 availability, temperature and light intensity. Cells irradiated with white light from a high pressure mercury lamp (from which the ultraviolet was eliminated) at the same energy, grow about eight times less rapidly. Besides an inefficient absorption ratio by the two photosynthetic pigment systems, the latter effect may be the result of the large fraction of blue light absorbed by xanthophylls. No energy transfer from these pigments to chlorophyll of either pigment system is found in blue-green algae[6].

We also found that a pigment system, containing both xanthophylls and the 745 nm pigment which occurs in *Anacystis*[7,8] could be precipitated from lamellae treated with sodium dodecyl sulphate (SDS). The fluorescence action spectrum of the 753 nm fluorescence band, emitted by the latter pigment form, shows bands in the carotenoid region, indicating some energy transfer between these non-

Fig. 1 Absorption spectrum of *Anacystis* grown for 2 d in a medium flushed with air + 5% CO_2 (. . . .) and plain air (- - -, × 12) at 35 °C and 1.2 10^4 lx from incandescent lamps. Continuous curve gives the absorption spectrum of yellow cells, obtained after reinoculation of exhausted growth medium with fresh cells and addition of 50% fresh medium.
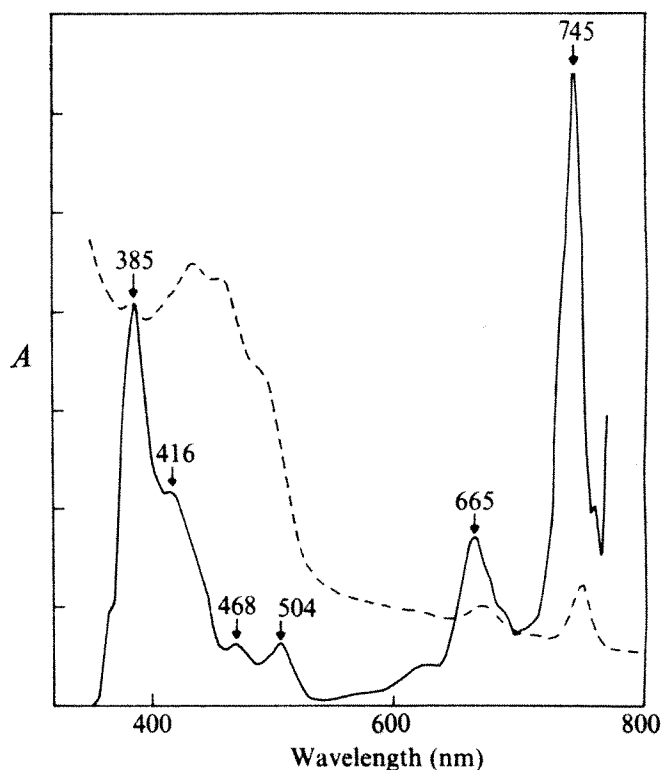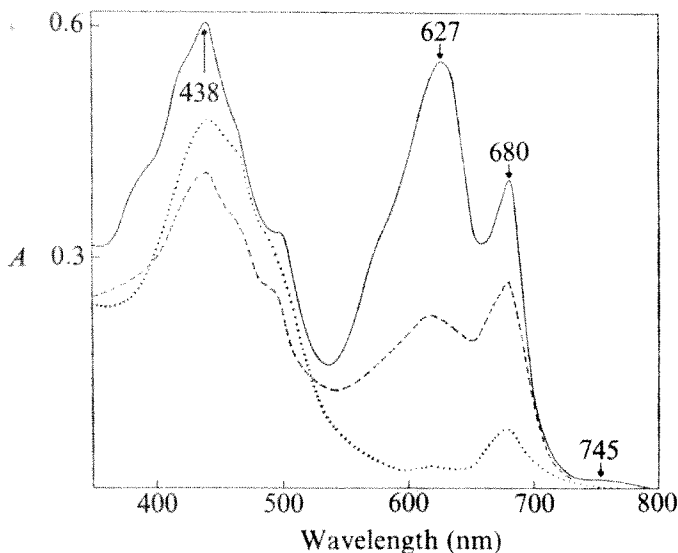
Fig. 2 Fluorescence action spectrum and absorption spectrum of a pigment complex isolated by SDS treatment from *Anacystis* cells which were disintegrated by the French press method. The complex emits a fluorescence band with a maximum at 753 nm. - - -, *A*; ———, fluorescence excitation.

fluorescent xanthophylls and fluorescing P745 (Fig. 2). The function of this pigment system in the energy scheme of this alga is not yet understood.

The growth pattern in the first few days after inoculation shows that, under favourable conditions of CO_2 availability, nutrients, temperature and spectral composition of the light, photosynthesis proceeds with much greater efficiency, even at high light intensities, than in the green alga *Chlorella*[9].

From the values measured during growth the conversion yield Q of electromagnetic into chemical energy can be calculated. An irradiation intensity of 50 W m^{-2} on a bottle with 200 cm^2 side area (volume 1 l) corresponds, after 1.5 d growth, to 3.6 kcalorie radiant energy taken up by the cells in 6 h (average A 700–400 75%). This illumination results in a cell production with a dry weight of 0.20 g l^{-1}. According to Oswald[10] the energy content of algae in general varies between 4.6 and 6.5 kcalorie g^{-1}, a value close to that derived from Rabinowitch[11]. If we take an average value of 5.5 kcalorie g^{-1}, *Anacystis* cells produced in 6 h, contain an energy equivalent to 1.1 kcalorie. From these values we obtain a conversion factor (energy efficiency) of $Q=0.30$. The latter is only an approximate value constant over most of the growth period and is of the same order of magnitude as that expected from the optimal values for photosynthesis measurements, corresponding to a quantum efficiency of eight and derived from short time experiments[11].

Our measurements indicate that *Anacystis* may be grown in conditions in which high intensity radiation is converted into chemical energy with a high yield.

J. C. GOEDHEER
J. W. KLEINEN HAMMANS

*Biophysics Research Group,
State University, Utrecht, The Netherlands*

1 Haldall, P., *Physiologia Pl.*, 11, 401–420 (1958).

2 Carr, N. G., and Whitton, B. A., *The Biology of Blue-Green Algae*, 360–367 (Blackwell, Oxford, 1973).
3 Fogg, G. E., Stewart, W. D. P., Fay, P., and Walsby, A. E., *The Blue-green Algae*, 129–142 (Academic, London, 1973).
4 Kratz, W., and Myers, J., *Am. J. Bot.*, **42**, 282–287, (1955).
5 Fogg, G. E., *Proc. R. Soc.*, **B139**, 372–397 (1952).
6 Goedheer, J. C., *Biochim. biophys. Acta*, **172**, 252–265 (1969).
7 Gassner, E. B., *Pl. Physiol. Lancaster*, **37**, 637–639 (1962).
8 Fischer, K., and Metzner, H., in *Progr. Photosynth. Res.*, **2**, (edit. by Metzner, H.), 547–551 (Tübingen, 1969).
9 Wassink, E. C., Kok, B., and Van Oorschot, J. L. P., in *Algal Culture, from Laboratory to Pilot Plants*, (edit. by Burlew, J. S.), 55–62 (Carn. Inst. Wash. Publ. no. 600, Washington, DC, 1953).
10 Oswald, W. J., in *Proc. Workshop Bio-Solar Conversion*, 38, (NSF/RANN report, 1973).
11 Rabinowitch, E., *Photosynthesis and Related Processes*, **1**, 49 and **2**, 1946 (Wiley, New York, 1945 and 1956).

Evidence for heterogeneity in heterochromatin of *Drosophila melanogaster*

IN *Drosophila melanogaster* the heterochromatin comprises the whole of the Y chromosome, about the proximal third of the X chromosome and the centromeric areas of chromosomes 2 and 3[1-4]. It appears throughout the cell cycle as darkly staining and highly condensed chromatin[2,3] and replicates later than euchromatin during the synthetic period[5]. The heterochromatin of *Drosophila* is considered genetically inert because it contains very few mappable genes, although it has marked genetic effects in determining the well known position effect[1]. It has recently been found that the heterochromatin of *Drosophila* corresponds to the C bands[6] and contains highly repetitive DNA[7-12]. None of these characteristics has, however, so far been of any use in solving the problem of the functional role of the heterochromatin. On the other hand, *Drosophila* offers unique possibilities for solving this problem, since in this organism it is possible to construct chromosomes containing different quantities of centromeric heterochromatin[4,13] and thus study the functions of this material. Obviously this type of approach requires a preliminary study on the possible heterogeneity of the heterochromatin. An initial result in this type of study has been obtained by showing that the heterochromatin regions of *D. melanogaster* fluoresce differently after staining both with quinacrine[14-16] and with the compound 33258 Hoechst[17]. Here we describe experiments in which the heterochromatin of *Drosophila* was differentiated by means of treatment of the living gangliar cells with 33258 Hoechst, which is known to decondense the centric heterochromatin of the mouse[18].

Use was made of third instar larvae of the Oregon stock and of a stock heterozygous for the translocation (2 ; 3) bw^{v4} (ref. 4). The nerve ganglia obtained from the dissection of the larvae were placed in a water solution containing NaCl (0.7%), foetal calf serum (20%) and 33258 Hoechst (40 μg ml^{-1}). After 2, 4, 6 and 8 h in this solution the ganglia were fixed and then squashed in acetic orcein[19,20]; 2 h before each fixation, colchicine was added to the culture solution, up to a final concentration of 10^{-4} M.

Figure 1 shows the effects of the treatment of the living gangliar cells with 33258 Hoechst. As may be seen, this treatment produces no alteration in the euchromatin, whereas it has a very marked effect on certain heterochromatic regions in which specific areas of decondensation are clearly visible. We emphasise that we observed no metaphases with chromosome aberrations or with heterochromatin decondensation patterns different from those shown in Fig. 1. The frequency of the various types of metaphase shown in Fig. 1 varies, however, with the fixation time (Table 1).

The data shown in Fig. 1 and Table 1 reveal that the heterochromatin of *D. melanogaster* responds in the following way to the treatment with 33258 Hoechst.
(1) The heterochromatin of the second chromosomes is not influenced.
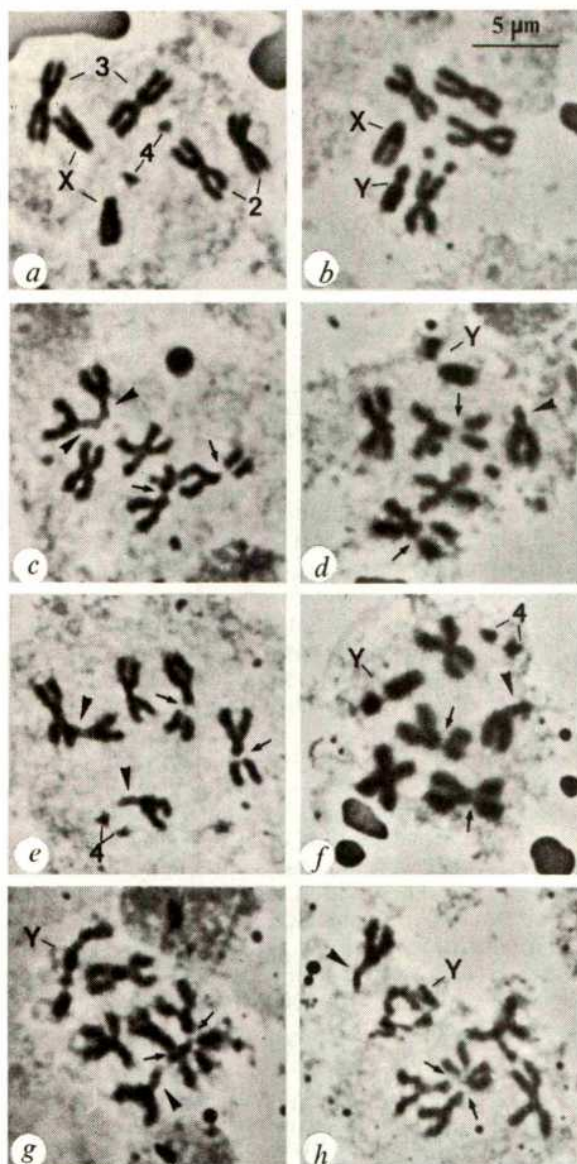
Fig. 1 The effect of 33258 Hoechst on the metaphase chromosomes of gangliar cells of *Drosophila melanogaster*: *a*, ♀ and *b*, ♂ normal non-treated cells; *c*, ♀ and *d*, ♂ cells treated for 4 h with H33258; *e*, ♀ and *f*, ♂ cells treated for 6 h with H33258; *g*, ♂ and *h*, ♂ cells treated for 8 h with H33258. In all the treated cells an area of drastic decondensation of the centromeric heterochromatin of a single arm of the 3rd chromosomes (↑) and the intermediate decondensation of part of the heterochromatin of the X chromosome (▲) can be seen. The male cells (*d*) and (*f*) show an area of drastic decondensation close to the centromere of the Y chromosome. In the male cells (*g*) and (*h*) the Y chromosome appears intermediately decondensed with the exception of some blocks of heterochromatin that remain compact. In the metaphases (*e*) and (*f*) the 4th chromosomes appear elongated and with the sister chromatids partially split. As may be seen the regions of drastic decondensation on the third chromosomes and on the Y chromosome seem to be isochromatid breaks. Under the microscope, however, it is often possible to observe very slender filaments of chromatin that connect the apparently broken chromatid ends.

(2) The heterochromatin of the right arm of the third chromosomes is not influenced. On the other hand, a distal portion with respect to the centromere of the heterochromatin of the left arm is drastically decondensed. The degree of decondensation of this heterochromatin portion does not vary with the time of fixation. The area of decondensation was assigned to the left arm of the third chromosome by using the stock heterozygous for the translocation (2 ; 3) bw^{v4} (Fig. 2).

Table 1 Effects of 33258 Hoechst on gangliar cells of *D. melanogaster* at various times of fixation after the treatment

Post-treatment time of fixation (h)	Sex	No. of cells scored	Normal cells (%)	Cells showing (%)				
				d.d.3 i.d.X	d.d.3 i.d.X d.d.Y.	d.d.3 i.d.X e.4	d.d.3 i.d.X d.d.Y e.4	d.d.3 i.d.X t.d.Y e.4
			(Fig. 1*a, b*)	(Fig. 1*c*)	(Fig. 1*d*)	(Fig. 1*e*)	(Fig. 1*f*)	(Fig. 1*g, h*)
2	♀♀	484	79.2	20.8	—	—	—	—
	♂♂	588	83.7	—	16.3	—	—	—
4	♀♀	179	48.0	52.0	—	—	—	—
	♂♂	616	49.8	—	50.2	—	—	—
6	♀♀	132	10.7	63.1	—	26.2	—	—
	♂♂	386	13.2	—	71.5	—	1.2	14.1
8	♀♀	153	—	20.8	—	79.2	—	—
	♂♂	132	—	—	12.6	—	2.3	85.1

Abbreviations: d.d.3=drastic decondensation on the third chromosomes; i.d.X=intermediate decondensation on the X chromosome; d.d.Y=drastic decondensation on the Y chromosome; e.4=fourth chromosomes elongated; t.d.Y.=total decondensation, except for certain regions, of the Y chromosome.

(3) Part of the heterochromatin of the X chromosome (presumably the greater part of that localised in XL) is not influenced; the remaining part, at all times of fixation, is seen to be intermediately decondensed.

(4) The Y chromosome, at all times of fixation, displays an area of drastic decondensation near the centromere. After 6–8 h of treatment it all appears intermediately decondensed with the exception of some blocks of heterochromatin which remain compact.

It is interesting to note that in the cells treated for 6–8 h the fourth chromosomes also appear elongated and with the sister chromatids split. We consider that this results from decondensation of heterochromatic material, since the fourth chromosomes, although they show no positive heteropycnosis in interphase[2,3], nevertheless display heterochromatic genetic properties[1], are late replicating[5] and positive in the C bands[6].

Taken as a whole, the present data indicate that, in relation to treatment with 33258 Hoechst, the heterochromatin of *D. melanogaster* can be divided into at least three types: that which is insensitive to this compound, that which is intermediately decondensed and that which is drastically decondensed. It is, however, possible that there are differences between the heterochromatin of the X chromosome, which decondenses right from the first fixation times, and that of the Y chromosome and the fourth chromosomes, which decondense after 6–8 h of treatment.

As regards the nature of the areas sensitive to the treatment with Hoechst, we believe that, in all probability, they contain DNA that is rich in AT. This is suggested by two considerations. First, the compound 33258 Hoechst decondenses all the mouse heterochromatic centric regions[18] in which we know that satellite DNA rich in AT is localised[21,22]. Second, all heterochromatic areas of the chromosomes of *Drosophila*, decondensed by the bibenzimidazole derivative correspond to areas that fluoresce brightly after staining with quinacrine[14–16] or 33258 Hoechst[17], which are both fluorescent probes specific for DNA rich in AT[17,23–25].

It is more difficult to establish the respective nature of the intermediately decondensed and the drastically decondensed regions. In principle, however, it is logical to assume that the degree of decondensation depends on the relative AT concentration in these regions. In this respect it could be suggested that in the intermediately decondensed regions are localised the two light satellites of *D. melanogaster*, which band at an approximate density of 1.690 g cm⁻³ in neutral CsCl (refs 8 and 10). The drastically decondensed regions, on the other hand, might contain the *Drosophila* d(A–T) rich satellite DNA[10,26,27] (1.675 (ref 26)—1.678 (ref 27)—1.672 (ref 10) g cm⁻³). In this respect it is interesting to note that all these light satellites have been shown, by *in situ* hybridisation, to have chromocentric localisation in the polytene chromosome preparations[8,10]. Futhermore the d(A–T) rich satellite DNA would be localised preferentially, though not exclusively, in the Y chromosome[26], which, as we have shown, contains a region of drastic decondensation.

S. Pimpinelli
M. Gatti
A. De Marco

Centro di Genetica Evolutiva del CNR,
Istituto di Genetica,
Università di Roma,
Rome, Italy

[1] Hannah, A., *Adv. Genet.* **4**, 87–121 (1951).
[2] Heitz, E., *Z. Zellforsch.* **20**, 237–287 (1933).

Fig. 2 Localisation on the left arm of the third chromosome of the region of drastic decondensation induced by 33258 Hoechst: nerve ganglia of larvae heterozygous for the translocation (2; 3) bw^v4 (points of breakage in 2R near bw and in 3L near centromere[4]) were fixed after 4h of treatment with H33258 (40 μg ml⁻¹). In the treated cells it may be clearly observed that the 3L arm translocated on to 2R shows the heterochromatic area drastically decondensed; however, the 3R arm is not influenced. *a*, ♀ and *b*, ♂ untreated cells heterozygous for t(2; 3) bw^v4; *c*, and *d*, ♂ the same type of cells fixed after 4 h of treatment with 33258 Hoechst.
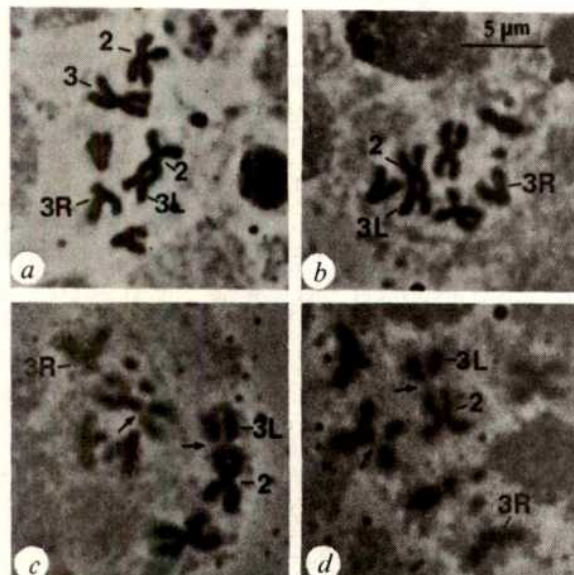
3 Kaufmann, B. P., *J Morph*, 56, 125–155 (1934).
4 Lindsley, D., and Grell, E H, *Carnegie Inst. Wash. Publ. No* 627 (1968)
5 Barigozzi, C., Dolfini, S , Fraccaro, M., Rezzonico-Raimondi, G., and Tiepolo, L., *Expl Cell Res.*, 43, 231–234 (1966)
6 Hsu, T. C, *J Hered*, 62, 285–287 (1971).
7 Botchan, M , Kram, R , Schmid, C. W., and Hearst, J. E., *Proc natn. Acad. Sci U.S.A*, 68, 1125–1129 (1971).
8 Gall, J. G , Cohen, F H., and Polan, M. D., *Chromosoma*, 33, 319–344 (1971).
9 Jones, K. W., and Robertson, F. W., *Chromosoma*, 31, 331–345 (1970).
10 Peacock, W J., *et al* , *Cold Spring Harb. Symp. quant Biol.*, 38, 405–416 (1973)
11 Perreault, W J , Kaufman, B P., and Gay, H., *Proc. natn. Acad. Sci. U.S.A.*, 70, 773–777 (1973).
12 Rae, P., *Proc natn. Acad Sci. U.S.A.*, 67, 1018–1025 (1970).
13 Lindsley, D L., *et al* , *Genetics*, 71, 157–184 (1972).
14 Adkisson, K P , Perreault, W J , and Gay, H., *Chromosoma*, 34, 190–205 (1971)
15 Faccio Dolfini, S , *Chromosoma*, 44, 383–391 (1974)
16 Vosa, C. G , *Chromosoma*, 31, 446–451 (1970)
17 Holmquist, G , *Chromosoma*, 49, 333–356 (1975)
18 Hilwig, I. and Gropp, A , *Expl Cell Res.*, 81, 474–477 (1973)
19 Gatti, M , Tanzarella, C , and Olivieri, G., *Nature*, 247, 151–152 (1974).
20 Gatti, M., Tanzarella, C., and Olivieri, G., *Genetics*, 77, 701–719 (1974)
21 Jones, K , *Nature*, 225, 912–915 (1970),
22 Pardue, M. L., and Gall, J. G , *Science*, 168, 1356–1358 (1970).
23 Ellison, J. R., and Barr, H J , *Chromosoma*, 36, 375–390 (1972).
24 Weisblum, B , and de Haseth, P. L., *Proc. natn Acad. Sci. U.S A.*, 69, 629–632 (1972)
25 Weisblum, B., and Haenssler, E., *Chromosoma*, 46, 255–260 (1974)
26 Blumenfeld, M., and Forrest, H. S , *Proc. natn Acad Sci. U.S.A.*, 68, 3145–3149 (1971).
27 Fansler, B. S., Travaglini, E. C , Loeb, L. A , and Schultz, J , *Biochem. biophys. Res. Commun.*, 40, 1266–1272 (1970)

Poly(U) polymerase in rat brain

MUCH interest has centred recently on the discoveries of ribohomopolymer sequences in RNAs of eukaryotic cells. Poly(A)$^{1-3}$, poly(U)^4 and poly(G)^5 regions have been found in nuclear and cytoplasmic RNA fractions. These sequences are thought to have special implications for regulation of biological functions of RNAs. It seems likely that these ribohomopolymer sequences are synthesised in a post-transcriptional process Poly(A) polymerases have been reported$^{6-7,13}$ and enzymatic activities catalysing short (two to five nucleotides) chains of poly(U) have been reported in cytoplasmic and microsomal fractions in rat liver^{8,9}. Enzymatic activities which polymerise four different ribohomopolymers independently have been found in nuclear ribonucleoprotein particles^{10}.

We have reported the presence of RNA-dependent RNA polymerase requiring four nucleoside triphosphates as substrate in an enzyme fraction of rat brain^{11} With this in mind, a poly(U) polymerase was separated from the same organ and was shown to have a primer requirement for RNAs of larger size, such as ribosomal RNAs (rRNAs), and viral RNA. The enzyme catalyses sequential polymerisation of poly(U) chains from the 3'-hydroxyl end of RNA. We report here studies on the isolation and characterisation of a new poly(U) polymerase. The activity of poly(U) polymerase was demonstrated in a supernatant which was solubilised with 0.4 M ammonium sulphate from the microsomal fraction (see legend to Table 1). This enzyme activity had an absolute requirement for a primer. The presence of creatine phosphate and creatine kinase stimulated the reaction, indicating that ribonucleoside phosphorylase is not involved and that ribonucleoside triphosphates rather than diphosphates are the actual substrates. Only slight incorporations, less than 10% of incorporation of ^{3}H-UMP, were demonstrated in the presence of other nucleoside triphosphates (^{3}H-ATP, ^{3}H-GTP and ^{3}H-CTP) as substrates (data not shown).

Primer specificity of the poly(U) polymerase was surveyed in the presence of several other primers (Table 2) rRNAs from rat brain, liver and *Escherichia coli* had higher activities than smaller RNAs in a transfer RNA (tRNA) fraction from the same sources. Kinetics of the synthesis showed that the efficiency of smaller RNA in a tRNA fraction as a primer was about 30% that of rRNA at any incubation time (Fig. 1). Qβ phage RNA also had a similar level of primer activity as rRNAs; but the primer activity of the fragmented Qβ phage RNA (4S) was the same as the activity of smaller RNAs in tRNA fraction. These results show the poly(U) polymerase preferred larger RNAs as a primer than smaller RNAs. The molecular

Table 1 Properties of poly(U) polymerase from rat brain

Assay conditions	^{3}H-UMP incorporated (pmol)
Complete system	26.8
+ DNase (10 µg)	27.9
+ RNase A (10 µg)	0
+ Actinomycin D (2 µg)	22.9
− Mg^{2+}	0.9
− Dithiothreitol	18.7
+ ATP, GTP, CTP (0.1 µmol each)	27.0
+ Phosphocreatine (1 µmol)+ creatine phosphokinase (10 µg)	34.9
− RNA	0

Reaction mixture contained in a final volume of 0.25 ml: 22 µmol Tris-HCl pH 8.3, 0.75 µmol MgCl$_2$, 0.125 µmol dithiothreitol, 0.1 µmol ^{3}H-UTP (2×10^6 c.p m. 0.1 µmol^{-1}), 5 µg 28S rRNA from newborn rat brain, 20 µg enzyme protein. Incubated at 36 °C for 30 min, stopped by addition of saturated pyrophosphate–sodium phosphate (1M), pH 7.4 (1:1) mixture and TCA. Enzymatic activity was measured by converting ^{3}H-labelled nucleoside monophosphates into acid-insoluble material. ^{3}H-labelled samples were counted in a liquid scintillation counter (Beckman LS 100) All procedures were done at 0 °C unless otherwise indicated. Wistar rats 8–12-weeks old were used. Fresh rat brain was homogenised in a glass-Teflon homogeniser with 10 volumes ice-cold buffer (10 mM Tris-HCl, pH 7.4, 15 mM KCl, 5 mM 2-mercaptoethanol). Mitochondria and cell debris were removed by centrifugation at 12,000g for 30 min. Supernatant was centrifuged at 105,000g for 60 min to obtain microsomal fraction. Microsomal pellet was suspended in 0.4 M ammonium sulphate solution, pH 7.4, and gently homogenised in a glass-Teflon homogeniser. After 60 min standing, microsomal fraction was centrifuged at 105,000g for 60 min. Supernatant was brought to 30% saturation by adding 100% saturated ammonium sulphate and left standing for 20 min. Resulting precipitate was removed by centrifugation at 17,000g for 10 min. The 30% saturated ammonium sulphate supernatant was brought to 45% saturation with solid ammonium sulphate and left standing for 20 min. Precipitate was collected by centrifugation at 17,000g for 10 min. Pellet was dissolved in SB (10 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$, 0.5 mM 2-mercaptoethanol) Protamin sulphate (30 µg ml^{-1}) was added to the dissolved pellet and was centrifuged at 17,000g for 10 min after 20 min of standing. An equal volume of 100% saturated ammonium sulphate was added to the supernatant, and after 20 min standing, pellet was collected by centrifugation at 17,000g for 10 min. Precipitate was dissolved in SB and 0.3 volumes glycerol was added to the medium. This enzyme fraction was stored at −60 °C.

weight of the tRNA is known to be about 20,000, and that of Qβ RNA 1,000,000 Considering the molecular sizes of tRNA and rRNA, tRNA should show higher activity at the same absorbance level, and this may also be true for

Fig. 1 Time course of reaction of poly(U) polymerase. Conditions of incubations were as in Table 1, except that the time of incubation varied O, 18S rRNA from rat brain; △, rat liver tRNA; □, minus RNA.
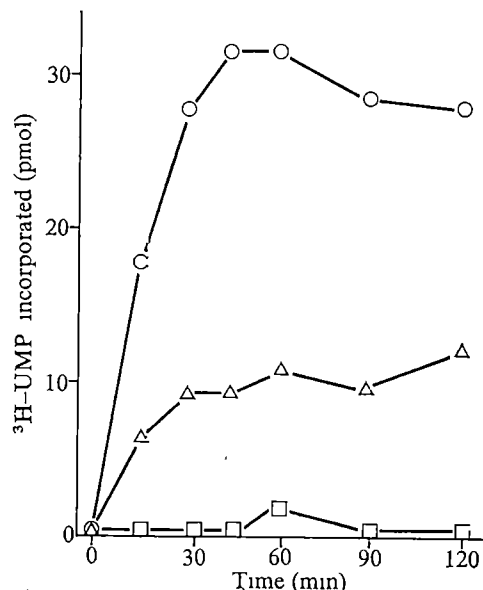
other ribohomopolymer polymerases[13]. We believe that poly(U) polymerase has a primer specificity for larger RNA molecules, that it has some important role in the regulation of metabolism of larger RNAs, such as mRNA, rRNA or heterogeneous nuclear RNA (HnRNA).

Native DNA could act as a primer for the synthesis, but denatured DNA could not. The poly(U) polymerase may be capable of serving native DNA as a primer at a reduced rate, or a different enzymatic entity in the poly(U) polymerase fraction using native DNA as a primer, may be active. These points may be clarified by further purification of the enzyme.

The average length of the newly incorporated poly(U) sequence was determined. Table 3 shows the chain length of the product increasing during the 45 min incubation period. The products formed by the poly(U) polymerase clearly indicate that the reaction is sequential polymerisa-

Table 3 Average chain length of polynucleotides synthesised *in vitro*

Incubation time (min)	Ratio of radioactivity $\left(\dfrac{\text{Nucleotide}}{\text{Nucleoside}}\right)$	Average chain length
15	7.65	9
45	19.12	20

Ribohomopolymers were synthesised using 28S rRNA from rat brain (5 μg per 0.25 ml) in 20-fold standard reaction mixtures at the time of incubation indicated and extracted with 0.1% SDS–phenol, precipitated with two volumes ethanol, dissolved in water and precipitated with 5% perchloric acid. Samples were then washed four times with 2.5% perchloric acid and finally with 66% ethanol–water. Alkaline hydrolysis was carried out with 0.33 N KOH at 36 °C for 18 h. Hydrolysed samples were chromatographed with standards of UMP and uridine on cellulose sheets (10×10 cm, Funakoshi) in a solution (isobutyric acid 13.2 ml, NH₃OH 0.2 ml, H₂O 6.6 ml). The appropriate areas were cut out and extracted with 0.01 N HCl. Radioactivity was then counted in a scintillation counter.

Table 2 Primer specificity of poly(U) polymerase

Primer	³H-UMP incorporated (pmol)	% Rate with *E. coli* rRNA
28S rRNA (rat brain)	32.2	77
18S rRNA (rat brain)	35.9	85
Rat liver rRNA	33.6	80
E. coli rRNA	42.0	100
Qβ phage RNA	33.0	79
Rat brain tRNA	21.8	52
Rat liver tRNA	13.4	32
E. coli tRNA	19.1	45
Fragmented Qβ phage RNA (4S)	14.7	35
Calf thymus DNA (native)	9.8	23
Calf thymus DNA (denatured)	0	0
Ribopolynucleotides		
Poly(U)	9.8	23
Poly(A)	13.4	32
Poly(C)	20.2	48
Poly(G)	0	0

Conditions of assay as described in Table 1, except that various nucleic acids were used as primers Each primer was tested at a concentration of 5 μg 0.25 ml⁻¹. Qβ phage RNA was prepared as described previously[12]. RNA was isolated from brains (cerebrum) of 3-d-old Wistar rats. Brain was cut up with scissors and stirred for 30 min at 4 °C in a solution containing 0.1 M Tris-HCl, pH 7.4, 10 mM MgCl₂, 0.5 mM 2-mercaptoethanol, 5 μg ml⁻¹ DNase This procedure was followed by adding 0.4% (w/v) sodium dodecyl sulphate (SDS) and incubating at 20 °C for 8 min. An equal volume of water-saturated phenol was added, and the mixture was shaken vigorously for 15 min at 4 °C. After centrifugation at 12,000g for 15 min, upper aqueous fraction was obtained. This phenol treatment was carried out four times, and extraction with ether was performed three times. The ether was flushed out with nitrogen gas, two volumes cold ethanol added; this was left to stand overnight at −20 °C. The mixture was centrifuged at 9,000g for 10 min, and the pellet was dissolved in TM (0.01 M Tris-HCl, pH 7.4, 5 mM MgCl₂). After ethanol precipitation, the pellet was again dissolved in TM. Supernatant after centrifugation at 12,000g for 20 min was used for further purification. RNA fraction was incubated with 20 μg ml⁻¹ DNase at 36 °C for 30 min and was purified by the SDS–phenol extraction method described above. To obtain rRNA and tRNA, extracted RNA fraction described above was layered on 25 ml 5–30% glycerol gradient (0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 5 mM EDTA) and ultracentrifuged in an SW 25.1 rotor at 21,000 r.p.m. for 18 h. Corresponding tRNA and rRNA fractions were collected by ethanol precipitation. RNAs from *E. coli* and rat liver were prepared as from brain. Fragmented Qβ phage RNA(4S) was prepared as follows. Intact Qβ phage RNA (1 mg ml⁻¹) was hydrolysed in 0.1 M KOH for various incubation times at 36 °C, and the hydrolysate adjusted to pH 7.0 with 2.5% HClO₄. Hydrolysate was then layered on 25 ml 5–30% glycerol gradient (0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 5 mM EDTA) and ultracentrifuged in an SW 25.1 rotor at 21,000 r.p.m. for 18 h. RNA sedimenting at the 4S position was collected by ethanol precipitation. To produce the new ends, the 4S RNA (0.36 mg ml⁻¹) was incubated at 36 °C for 4 min with sufficient alkaline phosphatase from *E. coli* to release 0.45 μmol p nitrophenol min⁻¹ as measured in the alkaline phosphatase assay described by Wilkie and Smellie[9]. Qβ phage RNA(4S) was prepared by SDS-phenol extraction method described above.

tion. Radioactivity of ribonucleoside 5′-phosphates (pppUp, ppUp or pUp) in the alkaline hydrolysate of radioactive products was not recovered, indicating that the poly(U) sequences were not synthesised *de novo* but formed by the successive addition of nucleotides to a pre-existing primer RNA.

To determine whether a sequential polymerisation occurs from the 3′-hydroxyl end of primer RNA or not, a nearest neighbour analysis using α-³²P-UTP was carried out. Qβ RNA and poly(C) were used as primer RNAs. Preparation of the products and thin-layer chromatography were carried out as described in the legend of Table 3. Using Qβ RNA, the ratio of radioactivity (UMP/(CMP+AMP)) increased during the 45 min incubation period from 5.92 (15 min) to 13.72 (45 min). At 45 min incubation, recovery of radioactivity in AMP, CMP and UMP was 4.6%, 2.2% and 93.3%, respectively. With poly(C), the ratio of radioactivity (UMP/CMP) also increased from 1.62 (15 min) to 3.20 (45 min). The 3′-hydroxyl end of Qβ RNA is known to be C–C–A (ref. 17) The recovery of radioactivity in CMP seems to be the result of removal of AMP during the preparation of Qβ RNA or incubation. This experiment clearly indicates that polymerisation occurs from 3′-hydroxyl end of RNA.

The precise biological implications of ribohomopolymer sequences in RNAs of eukaryotic cells remain obscure. It seems unlikely that the presence of poly(A) sequences is a prerequisite for translation[15]. It has recently been reported that mRNA having a poly(A) sequence on its 3′-end is more stable than poly(A)-free mRNA[16] It is known that HnRNAs containing poly(U) sequences are unstable compared with those containing poly(A)[4] The poly(U) polymerase catalysing sequential polymerisation from the 3′-hydroxyl end of RNA had a primer requirement for RNAs of larger size. We speculate that this enzyme has special significance in the regulation of metabolism of larger RNAs, such as mRNA, rRNA or HnRNA Wilkie and Smellie detected a poly(U) polymerase in the microsomal fraction of rat liver[8,9]. The enzyme, however, catalyses the synthesis of short (two to five nucleotides) chains of poly(U) and has no primer specificity, unlike our poly(U) polymerase. We are in the process of looking for poly(U) polymerases having similar properties in organs other than the brain.

NOBUMICHI HOZUMI
ICHIRO HARUNA
ITARU WATANABE

Department of Molecular Biology,

Katsuhiko Mikoshiba
Yasuzo Tsukada

*Department of Physiology,
School of Medicine, Keio University,
35 Shinanomachi, Shinjuku-ku, Tokyo, Japan*

1 Lim, L., and Canellakis, E. S., *Nature*, 227, 710–712 (1970).
2 Darnell, J. E., Wall, R., and Tushinski, R. J., *Proc. natn. Acad. Sci U.S.A.*, 68, 1321–1325 (1971).
3 Edmonds, M., Vaughan, M H., and Nakazato, H , *Proc. natn. Acad. Sci. U.S A*, 68, 1336–1340 (1971).
4 Burdon, R. H., and Shenkin, S , *FEBS Lett.*, 24, 11–14 (1972)
5 Lim, L., Canellakis, Z. N., and Canellakis, E. S , *Biochim. biophys. Acta*, 209, 112–127 (1970).
6 Niessing, J , and Sekeris, C E , *FEBS Lett* , 22, 83–88 (1972).
7 Winters, M. A , and Edmonds, M., *J. biol. Chem.*, 248, 4756–4762 (1973).
8 Wilkie, N M , and Smellie, R. M. S , *Biochem. J* , 109, 485–494 (1968)
9 Wilkie, N M., and Smellie, R M. S , *Biochem. J* , 109, 229–238 (1968).
10 Niessing, J., and Sekeris, C. E., *Nature new Biol* , 243, 9–12 (1973).
11 Mikoshiba, K., Tsukada, Y., Haruna, I., and Watanabe, I., *Nature*, 249, 445–448 (1974).
19 Haruna, I , and Spiegelman, S , *Proc natn Acad Sci. U.S A.*, 54, 579–587 (1965).
13 Burkard, G , and Keller, E B., *Proc. natn. Acad Sci U S A* , 71, 389–393 (1974)
14 Adesnik, M , Salditt, M , Thomas, W., and Darnell, J E., *J. molec Biol.*, 71, 21–30 (1972)
15 Williamson, R., Crossley, J , and Humphries, S., *Biochemistry*, 13, 703–707 (1974).
16 Huez, G , *et al* , *Proc natn. Acad. Sci U S A.*, 71, 3143–3146 (1974)
17 Goodman, H M , Billeter, M A., Hindley, J., and Weissmann, C , *Proc. natn Acad. Sci. U.S.A.*, 67, 921–928 (1970).

N-terminal sequences of secretory piece and of α chains of different allotype in rabbit secretory IgA

The translocation hypothesis[1,2] states that in the process of antibody synthesis any one of a number of immunoglobulin heavy chain V genes is available for union with any of the class-specific C genes. Whereas almost all structural data support the widely held view that all V genes are able to translocate to any of the class-specific C genes, some data suggest that this may not be the case. Wilkinson[3] found the N-terminal sequence PCA–Ser–Ser (PCA, pyrrolidone carboxylic acid) to be present in a rabbit α chain preparation but absent in pooled rabbit γ chains. Other workers[4,5] have described an N-terminal heavy chain sequence apparently confined to the IgM class of human immunoglobulins.

We have reinvestigated the former result in view of the finding that secretory piece (SP), a polypeptide chain present in secretory IgA (sIgA) but absent from IgG, has a molecular weight of 60,000–70,000 (refs 6 and 7), in both the native and fully reduced state, rather than being a disulphide-linked dimer with subunits of molecular weight 25,000 (ref. 8). Wilkinson isolated his α chain preparation after complete reduction of rabbit sIgA using gel filtration with Sephadex G-200; secretory piece could have contaminated this preparation and provided the novel N-terminal peptide The fact that the yield of the PCA–Ser–Ser peptide from the α chain preparation was 14–16% (ref. 3) and that the ratio of α chain to SP in the intact molecule is 4:1 (ref. 7) suggested that the above reasoning may be correct.

Wilkinson[3] compared the N-terminal PCA peptides of rabbit α chains of allotype a1 and a3 with peptides isolated in an earlier study of γ chains of the two allotypes[9]. The *a* locus allotypic specificities are located in the V region of rabbit heavy chains of all the known classes and have been shown for γ chains to be associated with multiple differences in primary structure[9,10]. Differences seen in the N-terminal PCA peptides of rabbit γ chain[9] (see Table 1) of the two allotypes proved similar in α chains of different allotype except for the additional peptide PCA–Ser–Ser found in α chain of both a1 and a3 specificity in a yield of 0 15 mol mol⁻¹.

In this study, preliminary experiments on totally reduced and alkylated sIgA showed, by alkaline urea and SDS gel electrophoresis, that the α chain fraction was indeed contaminated by secretory piece. We therefore tried to separate the α chain fraction from secretory piece. sIgA was isolated from clarified rabbit colostrum using the method of Cebra and Robbins[11] with slight modification. Attempts to separate α chain from SP after complete reduction and alkylation of sIgA, either by gel filtration, preparative gel electrophoresis or immunoadsorbents, proved fruitless (A.P.J. and L.E.M., unpublished). We purified secretory piece by taking advantage of the fact that not all SP is covalently bound to the sIgA molecule[8]. Thus, in our preparation, gel filtration in 3 M guanidine[8] released about 50% of the SP from sIgA; this fraction was pure on SDS gel electrophoresis and was free of any light chain contamination as judged by anti-light chain antisera. The N-terminal half of the α chain was eventually freed of the remaining covalently bound SP by peptic digestion of sIgA for 20 h at 37 °C in 0.1 M glycine-HCl pH 2.4 (A P J. and L.E.M., unpublished) followed by gel filtration on G-200 in 0.1 M phosphate buffer, pH 6.8. Such treatment digested SP and the α chain Fc region into peptides releasing a large fragment, $F(ab')_{2\alpha}$, equivalent in component structure to the well defined $F(ab')_2$ fragment released by peptic digestion of IgG (ref. 12). The $F(ab')_{2\alpha}$ fraction was unreactive with antisera to SP.

The isolated SP and $F(ab')_{2\alpha}$ from both allotypes a1 and a3 were completely reduced and alkylated, digested with Pronase and the PCA peptides collected in the unretarded fraction from Dowex 50 (×2; H⁺ form). Purification of the

Table 1 PCA peptides of Aa1, Aa3 $F(ab')_{2\alpha}$ and SP*

| | γ chain[9] | | α chain | | SP |
	Aa1	Aa3	Aa1	Aa3	
PCA–Ser–Val–Glu	0.56	—	0.61	—	—
PCA–Ser–Leu–Glu	0 14	0.50	0.15	0.43	—
PCA–Glu–Gln	—	0.26	—	0.27	—
PCA–Ser–Ser	—	—	—	—	0.65
Total	0.70	0.76	0.76	0.70	0.65

Yields as mol peptide per mol polypeptide chain.

PCA peptides from the Dowex 50 fractions was carried out using gel filtration and paper electrophoresis as described by Wilkinson[9].

The N-terminal PCA peptides isolated from SP, Aa1 $F(ab')_{2\alpha}$ and Aa3 $F(ab')_{2\alpha}$, are described in Table 1 and compared with the γ chain peptides described previously[9]. Peptide yields were calculated using the same assumptions used in the earlier studies[3,9]. The yields of the peptides PCA–Ser–Val–Glu and PCA–Ser–Leu–Glu (Table 1) from the $F(ab')_{2\alpha}$ fragment of a1 allotype proved similar to those derived from γ chain as did the yields of peptides PCA–Ser–Leu–Glu and PCA–Glu–Gln from Aa3 $F(ab')_{2\alpha}$ (ref. 9). There was no indication of the peptide PCA–Ser–Ser in either $F(ab')_{2\alpha}$ digest; but this peptide was isolated in good yield (65%) from the SP digest (Table 1). Although, unlike Wilkinson[3], we digested light chains together with the N-terminal region of α chain, we isolated no PCA peptides other than those normally found in γ chain. This reflects the fact that the vast majority of the rabbit light chain fraction is of the unblocked K type.

We conclude that the N-terminal sequence of rabbit secretory piece is PCA–Ser–Ser and that the earlier report[3] that this represented a sequence unique to rabbit α chain V region was incorrectly made because of the inability to separate these two polypeptide chains before characterisation Although our finding does not rule out the possibility that some V region subgroups may be confined to a certain

class of heavy chain it does remove some structural evidence against a simple translocation hypothesis.

We thank Professor R. R. Porter for discussion and Mr A. C. Willis for technical assistance.

ALAN P. JOHNSTONE
LAWRENCE E. MOLE

MRC Immunochemistry Unit,
Department of Biochemistry,
University of Oxford, Oxford OX1 3QU, UK

1 Dreyer, W. J., and Bennett, J. C., *Proc. natn. Acad. Sci. U.S.A.*, 54, 864–869 (1965).
2 Gally, J. A., and Edelman, G. M., *Nature*, 227, 341–348 (1970).
3 Wilkinson, J. M., *Nature*, 223, 616–617 (1969).
4 Bennett, J. C., *Biochemistry*, 7, 3340–3344 (1968).
5 Kohler, H., Shimizu, A., Paul, C., Moore, V., and Putnam, F. W., *Nature*, 227, 1318–1320 (1970).
6 Halpern, M. S , and Koshland, M. E., *Nature*, 228, 1276–1278 (1970).
7 O'Daly, J. A., and Cebra, J. J., *Biochemistry*, 10, 3843–50 (1971)
8 Cebra, J. J., and Small, P. A , *Biochemistry*, 6, 503–512 (1967).
9 Wilkinson, J. M., *Biochem. J.*, 112, 173–185 (1969)
10 Mole, L. E., Jackson, S. A., Porter, R. R., and Wilkinson, J. M., *Biochem. J.*, 124, 301–318 (1971).
11 Cebra, J. J., and Robbins, J. B., *J. Immun.*, 97, 12–24 (1966).
12 Nisonoff, A., and Dixon, D. J., *Biochemistry*, 3, 1338–1342 (1964).

Possible role for poly(A) as an inhibitor of endonuclease activity in eukaryotic cells

A NUMBER of laboratories have reported the isolation from eukaryotic cells of mRNAs linked covalently at their 3'-OH termini to polyadenylic acid (poly(A))[1–4]. The chain of adenylic acid residues has been found also at the 3' termini of RNAs isolated from several well characterised viruses[5–7]. Although considerable evidence exists to suggest that the insertion of poly(A) on the RNA chains destined to become mRNA is a post-transcriptional event[8], there is at present little understanding of the part poly(A) plays in the subsequent stages of protein synthesis.

In this communication we should like to suggest a possible role for the polynucleotide in maintaining the structural and functional integrity of mRNA. The role is based on the observation that poly(A) can act as a general inhibitor of endonuclease activity and in so doing can prevent the degradation of mRNA in the cytoplasm. The enzymes used in this study have been isolated from microbial and from human sources. Their purification and properties have been described elsewhere[9–11].

Poly(A), much like the ordered polynucleotide, polyguanylic acid (poly(G)), was found to act as a strong inhibitor of a microbial ribonuclease isolated from *Citrobacter* sp.[9,12]. The inhibition of enzyme activity induced by poly(G) can be only partially reversed by increasing concentrations of buffer. In contrast, the inhibition induced by poly(A) is strongly dependent on the ionic strength of the buffering mediums (Fig. 1). As the molar concentration of buffer is increased, the inhibitory effect of poly(A) is · reduced until, at the highest buffer concentration studied, the presence of the polynucleotide has relatively little effect on enzyme activity (Fig. 1). On the other hand, poly(G), in the highest molarity buffer, remains a powerful enzyme inhibitor.

Since there is considerable variation in the number of nucleotide residues in poly(A) at the end of individual mRNAs[14], it was of interest to study the relationship between chain length and the inhibitory properties of the polynucleotide. As shown in Fig. 2, the two shortest chains with an average monomer length of 25 (25) and 45 (45) residues respectively inhibited enzyme activity to essentially the same degree (I_{50}=4 5×10^{-6} M). The intermediate-sized polymer with an average monomer length of 90 residues
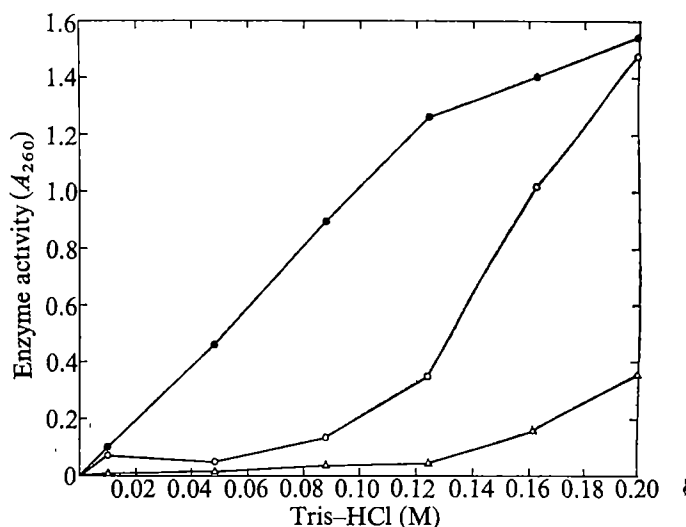
Fig. 1 Reversal by increasing concentrations of Tris-HCl buffer of polynucleotide-induced inhibition of *Citrobacter* RNase activity. Enzyme activity was measured in 1 ml of a reaction mixture consisting of varying concentrations of Tris-HCl buffer (pH 7.1), 0.5 μmol of poly(U) and 15 U of enzyme. After incubation for 5 min at 37 °C, the reaction was stopped by the addition of 1 ml of 2 N perchloric acid containing 20 mM lanthanum nitrate and the reaction vessel was placed in an ice bath for 20 min. The mixture was then clarified by centrifugation and the A_{260} of the acid-soluble nucleotides measured[13]. When the effects of the polynucleotide on enzyme activity were to be studied, poly(A) (5×10^{-6} M) and poly(G) (2.5×10^{-6} M) were added, before the addition of enzyme, to separate reaction mixtures. RNase activity measured in a control vessel (●) and in the presence of poly(A) (○) and poly(G) (△).

(90) was a better inhibitor (I_{50}=2.9×10^{-6} M) than either of the smaller chains. Finally, the polymer having the highest molecular weight (average monomer length of 315) proved to be the best inhibitor of all (I_{50}=2.1×10^{-6} M). Although greater inhibition of enzyme activity was observed as the average chain length of poly(A) was lengthened, the increase was gradual and did not seem to be so strongly controlled by chain length that inhibition was a step function of average molecular weight in the region available for study. The gradual decrease in inhibitory properties associated with decreasing chain length suggested that below a certain size, inhibition by poly(A) would not occur. The

Fig. 2 Inhibition of *Citrobacter* RNase activity by various molecular weight fractions of poly(A). Average monomer length (Miles Laboratories) based on intrinsic viscosity and sedimentation velocity, 50% range: □, 25 (25–51); △, 45 (39–82); ○, 90 (62–108); ●, >300. Enzyme activity was measured as in Fig. 1, except that 55 μmol of Tris-HCl buffer (pH 7) was used in all the determinations
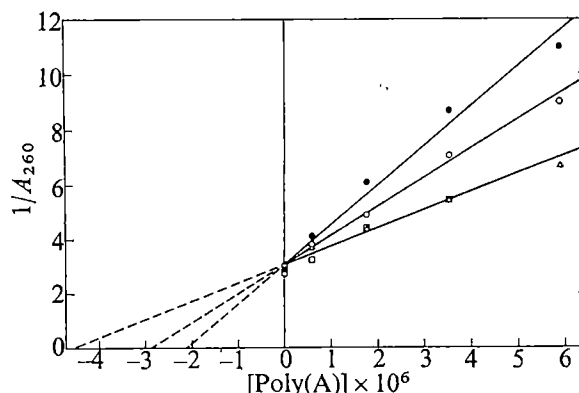
Table 1 Reversal by polyamines of poly(A)-induced inhibition of RNase activity

RNase	Concentration of poly(A)	—	MgCl₂	Enzyme activity (ΔA_{260}) of cation added Putrescine	Spermidine	Spermine
Citrobacter	—	1.31	1.35	1.29	1.52	1.49
	5.0×10^{-6}M	0.29	0.30	0.33	1.25	1.50
Human plasma	—	2.10	2.27	2.39	2.47	2.26
	1.5×10^{-5}M	0.78	0.78	0.97	2.23	2.10
Human spleen	—	1.05	1.11	1.38	1.43	1.21
	2.0×10^{-6}M	0.08	0.78	0.50	1.44	1.27

Citrobacter RNase activity was measured as in Fig. 1, except that 40 μmol of potassium phosphate buffer (pH 7.1), were used in place of Tris-HCl. When the effects on enzyme activity of poly(A) and/or cations listed above were studied, 0.005 μmol of poly(A), followed by 0.5 μmol of cation were added before the addition of enzyme to reaction mixtures similar to those described above. The hydrolysis of poly(C)(1.5 μmol) used as a measure of the activity of human plasma RNase was assayed as described elsewhere[10]. Here also poly(A) (0.015 μmol) and 0.5 μmol of cation were added to the reaction mixtures before the addition of 21 U of enzyme. The human spleen RNase activity[11] was measured in a reaction mixture (1 ml) containing 1 μmol of potassium phosphate buffer (pH 6.2), 40 μmol of poly(U) and 10 U of human spleen RNase. After incubation for 15 min at 37 °C, enzyme activity was measured by the production of acid-soluble nucleotides[14]. Poly(A) (0.002 μmol) and cations (0.4 μmol) were added also to reaction mixtures before the addition of enzyme.

critical chain size could not be determined, however, because of the lack of availability of a graduated series of smaller polymer chains.

Another characteristic of polynucleotide inhibition of RNase activity is that it is readily reversible when the highly charged polyamines are added. As is apparent from Table 1, the poly(A)-induced inhibition of a number of RNases can be reversed with these substances. At concentrations of poly(A) sufficient to cause severe reductions in enzyme activity of both the *Citrobacter* and a human plasma RNase, only spermine or spermidine could restore activity to uninhibited levels. Putrescine and a variety of metal cations tested (represented by Mg²⁺ in Table 1) were ineffective in reversing the inhibition. Enzyme activity of a third RNase (from human spleen), although completely inhibited by poly(A), could be restored by spermine or

spermidine, and to a lesser extent by Mg²⁺ and putrescine.

The influence of poly(A) on RNase activity was of interest from the point of view of enzyme stability as well. The association between polynucleotide and enzyme was found to protect the RNase from thermal inactivation. Thus, in the absence of the polynucleotide, the half life of a human liver RNase (unpublished results) was 20 min (Fig. 3), whereas in the presence of poly(A), the half life of the enzyme was found to be increased by as much as fivefold.

The results of these studies suggest a model whereby poly(A), at the terminus of a mRNA, acts to prevent the degradation of the mRNA. The polynucleotide would do this by binding to, and thus inhibiting, any RNase in the vicinity of the mRNA. With this in mind, it is entirely conceivable that translation can occur with an RNase bound to the poly(A) moiety at the end of the mRNA chain. This would also help explain the long lived mRNAs of eukaryotic cells in contrast to the rapidly degraded ones of prokaryotes[15]. Degradation of the mRNA can, nonetheless, be assured by any of a number of means that would dissociate the complex formed between the mRNA-poly(A) and the RNase. An increase in ionic strength or in polyamine concentration would, of course, be examples of factors causing dissociation of the complex and thereby reversing the inhibition of the enzymes. The association between RNase and polynucleotide also provides a means of storing the enzyme for long periods, thus ensuring that the protein, although quiescent, is not denatured and is immediately active when needed. In effect, the mRNA-poly(A) chain might carry its own "self-destruct" mechanism. Studies are in progress attempting to verify the model *in vivo*.

Fig. 3 The effect of poly(A) on the thermal inactivation of human liver RNase. To one of two flasks, each containing 40 U of human liver RNase in 0.1 M potassium phosphate buffer (pH 6.4), 0.25 μmol of poly(A) were added. The final volume in each vessel was brought to 1 ml and the mixtures incubated at 37 °C. Aliquots (25 μl) withdrawn from each vessel at 5-min intervals were tested for enzyme activity in a reaction mixture containing 0.25 mg of yeast RNA and 100 μmol of potassium phosphate buffer (pH 6.4)[11]. After 15 min at 37 °C, the reaction was stopped by the addition of 1 ml of 2 N perchloric acid and the mixture chilled for 20 min. The A_{260} of acid-soluble nucleotides was measured after clarification of the mixture by centrifugation. Enzyme activity (●); enzyme activity in presence of poly(A) (○).

CARL C. LEVY   PHILLIP B. JEWETT
MORTON SCHMUKLER   PHILIP A. HIETER
JOSEPH J. FRANK   STEPHEN M. LEGENDRE
TIMOTHY P. KARPETSKY   ROBERT G. DORR

Enzymology and Drug Metabolism Section,
Laboratory of Pharmacology,
Baltimore Cancer Research Center,
National Cancer Institute,
National Institute of Health,
Baltimore, Maryland 21211

1 Kates, J., *Cold Spring Harb. Symp. quant. Biol.*, **35**, 743–762 (1970).
2 Mendecki, J., Lee, S.-Y., and Brawerman, G., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 1331–1335 (1971).
3 Edmonds, M., Vaughan, M. H., and Nakazato, H., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 1336–1340 (1971).
4 Darnell, J. E., Wall, R., and Tushinski, R. J., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 1321–1325 (1971).

5 Perlman, S., Abelson, H. T., and Penman, S., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 350–353 (1973).
6 El Manna, M. M., and Bruening, G., *Virology*, **56**, 198–206 (1973).
7 Spector, D. H., and Baltimore, D., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 2983–2987 (1974).
8 Adesnik, M., Salditt, M., Thomas, W., and Darnell, J. E., *J. molec. Biol.*, **71**, 21–30 (1972).
9 Levy, C. C., Mitch, W. E., and Schmukler, M., *J. biol. Chem*, **248**, 5712–5719 (1973).
10 Schmukler, M., Jewett, P. B., and Levy, C. C., *J. biol. Chem.*, **250**, 2206–2212 (1975).
11 Delaney, R., *Biochemistry*, **2**, 438–444 (1963).
12 Levy, C. C., Hieter, P. A., and LeGendre, S. M., *J. biol. Chem.*, **249**, 6782–6789 (1974).
13 Kalnitsky, G., Hummel, J. P., and Dierks, C. J., *J. biol. Chem.*, **234**, 1512–1516 (1959).
14 Brawerman, G., *A. Rev. Biochem.*, **43**, 621–642 (1974).
15 Morse, D. E., Mosteller, R., Baker, R. F., and Yanofsky, C., *Nature*, **223**, 40–43 (1969).

Stereoselective blockade of the dopamine receptor and the X-ray structures of α and β-flupenthixol

THE introduction of neuroleptics of the phenothiazine and thioxanthene classes has revolutionised the treatment of schizophrenia and there is increasing evidence[1-3] that links this antipsychotic action to a blockade of dopamine receptors in the brain. The thioxanthene compounds are of particular interest as, because of the presence of an exocyclic double bond and a substituent in the tricyclic nucleus, they exist as *cis–trans* geometric isomers about the double bond with respect to the ring substituent (Fig. 1). Virtually all the neuroleptic activity is confined to the *cis* isomer[4].

One of the most potent drugs in this group is flupenthixol. (Fig. 1); a mixture of the *cis* (α) and *trans* (β) isomers is a clinically efficaceous antipsychotic agent[5] but in dopamine receptor related animal tests the α isomer is much more potent than the β form[4] (Table 1). In *in vitro* studies using the dopamine sensitive adenyl cyclase system of the rat corpus striatum as a model of the dopamine receptor, the α isomer is a very potent blocker of the dopamine induced enzyme stimulation whereas the β isomer is almost inactive[6] (Table 1).

In an attempt to explain how chlorpromazine is able to block dopamine receptors, attention has been focused on a possible spatial complementary relationship between certain

portions of the crystal structure of chlorpromazine and dopamine[1]. Further support for this concept has been obtained from a detailed conformational analysis of 15 drugs of the tricyclic class[7]. As flupenthixol displays such a marked degree of stereoselectivity in blocking dopamine receptors, it is believed that X-ray analysis of the two isomers could provide additional insight into the various interatomic distance and overall conformational requirements for effective dopamine receptor antagonism. Although the *cis* and *trans* geometrical isomers will have differing physical properties this is unlikely to be the sole factor accounting for their differential pharmacological potency, as neuroleptics possessing optically active centres, such as butaclamol, also show a high degree of stereoselectivity[7-9].

Crystals of each isomer were obtained and the cell dimensions and other related parameters for each compound were found:

α-flupenthixol: orthorhombic; *Pbca*; $a = 8.827$, $b = 20.048$, $c = 20.251$ Å; 8 molecules per cell; final R factor 0.066 for 1,094 reflections.
β-flupenthixol: monoclinic; $P2_1/c$; $a = 9.011$, $b = 15.218$, $c = 18.493$ Å; $\beta = 120.37°$; 4 molecules per cell; current R factor 0.072 for 2,308 reflections.

Diffractometer data for both compounds were collected using Cu-K_α radiation to a resolution of 0.89 Å ($2\theta_{max}$ of 120°) and each structure was solved by the application of locally programmed (G. M. Sheldrick, unpublished) multisolution direct method techniques. Complete details of these structures will be published elsewhere. Figure 1 shows a general view of each isomer based on approximately equivalent orientations of the tricyclic nucleus. The *cis* or *trans* configuration at the exocyclic double bond is clearly apparent and each side chain exhibits markedly different conformation although the piperazine rings favour the –CF$_3$ 'side' of the molecule in either case. Principal torsion angles for the side chain are shown in Fig. 1. The dihedral angles between the aromatic ring mean planes are 152° and 143° for α and β-flupenthixol respectively.

Various lines of evidence support the concept that the preferred conformation of dopamine at its receptor site is the fully extended *trans* form[7]; therefore, it has been suggested that the binding site for dopamine's amino group is about 5.1 Å from the centre of the aromatic ring[7]. There is also evidence that the dopamine receptor antagonism by the neuroleptics is
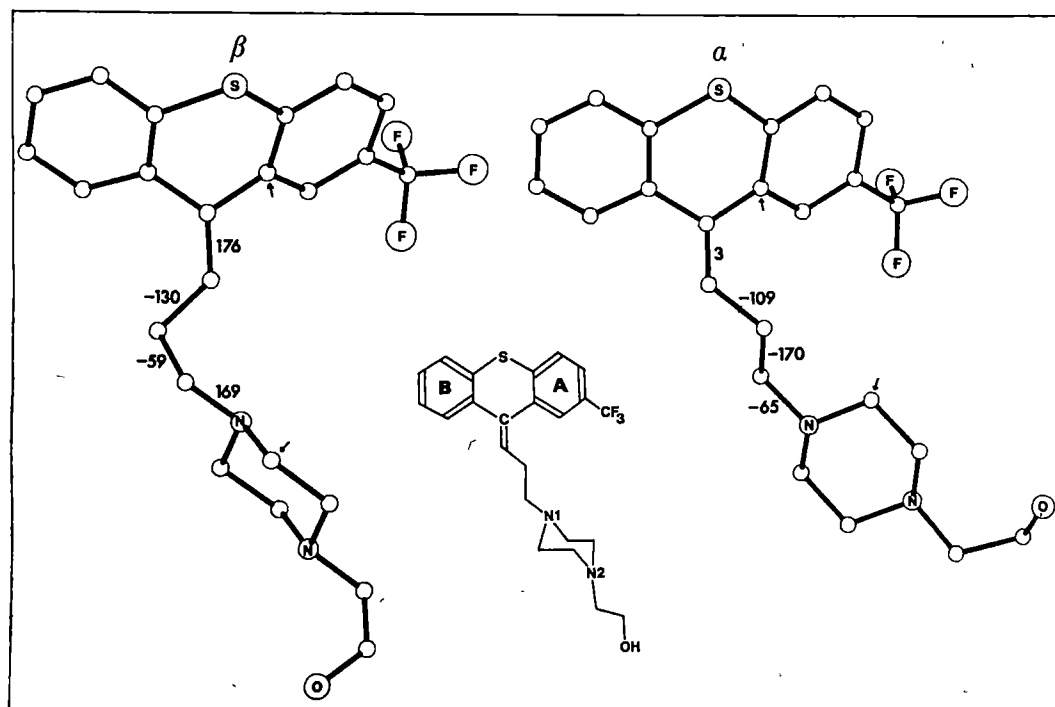
Fig. 1 α and β isomers of flupenthixol computed from the crystallographic coordinates, together with a chemical representation of the molecules (shown in the α form) on which is indicated labelling referred to in the text. The values of some principal torsion angles (in degrees) for each isomer are shown alongside the bond to which they refer, and where an ambiguity in definition occurs, an arrow marks atoms used in the calculations.

Table 1 Comparison of the effects of α and β-flupenthixol on various dopamine receptor systems

	Antagonism of apomorphine induced stereotypy in rats ED_{50} (mg kg^{-1}) (ref. 4)	Antagonism of amphetamine induced stereotypy in rats ED_{50} (mg kg^{-1}) (ref. 4)	Ki for dopamine sensitive adenyl cyclase antagonism[6]
α-flupenthixol	0.3	0.07	1.0×10^{-9} (M)
β-flupenthixol	> 80	> 160	$> 5 \times 10^{-6}$ (M)

competitive[10,11] and for simplicity this can be considered to indicate occupation, by the neuroleptic, of the same site as the natural agonist. Thus it is of interest to contrast significant intramolecular distances between α and β-flupenthixol. In α-flupenthixol the distances of N1 from the centres of the two aromatic rings A and B (Fig. 1) are 5.82 Å and 7.46 Å respectively, and the corresponding distances from N2 are much longer, with N2–A = 7.75 Å and N2–B = 10.26 Å.

This suggests that N1 could bind at the receptor site normally occupied by the amino group of dopamine whereas the aromatic ring A would interact at the site normally influenced by the benzene ring of dopamine. The importance of N1 rather than N2 as the atom involved in the antagonistic binding is supported by comparison with similar distances found in α-chlorprothixene[7,12], a pharmacologically active compound which, however, has only a single N atom in the side chain. There is a marked difference in the almost inactive β-flupenthixol where N1–A = 6.09 Å, N1–B = 6.46 Å, N2–A = 8.24 Å and N2–B = 9.30 Å, indicating that the α-isomer exhibits a better 'fit' as a dopamine antagonist. Extrapolation from results of crystal structure determinations to the conformation of molecules in physiological conditions has always to be interpreted with caution, but the absence of any unusually close intermolecular contacts in the crystal structures suggests that the observed conformation is not the result of packing forces and that a closely similar conformation could be found in vivo.

The A and B rings of the tricyclic nucleus are differentiated by the presence of the –CF$_3$ moiety. This group is of evident importance for pharmacological activity although its mode of action is not clearly understood. It is perhaps of interest to note that the molecular asymmetry introduced in the tricyclic nucleus by the –CF$_3$ group is enhanced in the α form where the difference in the distance of N1 from the centres of the aromatic rings is greater than in the β form[7].

The X-ray studies reported here were carried out on the free bases. An X-ray analysis of the dihydrochloride salts is in progress to investigate the effect of positive charges on the nitrogen atoms of the piperazine ring on the conformation of these molecules.

We thank the MRC for financial support and the SRC for provision of the diffractometer. Olga Kennard is on the External Staff of the MRC. Dr I. Moller-Nielsen of H. Lundbeck and Co., Denmark, provided the specimen crystals.

MICHAEL L. POST
OLGA KENNARD

University Chemical Laboratory,
Lensfield Road, Cambridge CB2 1EW, UK

ALAN S. HORN

Neurochemical Pharmacology Unit,
Department of Pharmacology,
Medical School, Hills Road, Cambridge, UK

1 Horn, A. S, and Snyder, S. H., *Proc. natn. Acad. Sci. U.S.A.*, 68, 2325–2328 (1971).
2 Matthysse, S., *Fedn Proc.*, 32, 200–205 (1973)
3 Snyder, S. H., Banerjee, S. P., Yamamura, H. I., and Greenburg, D., *Science*, 184, 1243–1253 (1974).
4 Moller-Nielsen, I., *et al.*, *Acta pharmac. tox.*, 33, 353–362 (1973)
5 Gottfries, C. G., *Br. J. Psychiat.*, 119, 547–548 (1971).

6 Miller, R. J., Horn, A. S., and Iversen, L. L., *Mol. Pharmac.*, 10, 759–766 (1974)
7 Horn, A. S., Post, M. L., and Kennard, O., *J. Pharm. Pharmac.* (in the press).
8 Miller, R. J., Horn, A. S., and Iversen, L. L., *J. Pharm. Pharmac*, 27, 212–213 (1975).
9 Bruderlein, F. T., Humber, L. G, and Voith, K, *J. med. Chem.*, 18, 185–188 (1975).
10 York, D. H., *Brain Res.*, 37, 91–99 (1972).
11 Clement-Cormier, Y. C., Kebebian, J W., Petzold, G. L., and Greengard, P., *Proc. natn. Acad Sci. U.S.A.*, 71, 1113–1117 (1974).
12 Post, M. L., Kennard, O., and Horn, A. S, *Acta crystallogr.* B30, 1644–1646 (1974).

Alteration of cavity geometry by structural modification of clathrate host molecules

IN the course of systematic studies[1] of the synthesis and properties of clathrate inclusion compounds we found that compound II, formed by adding a methyl group to the clathrating compound I, also exhibits clathrating properties. It was evident, however, both from preliminary crystallographic measurements and from their very different selectivities towards guest molecules that the cavities in the host structure of I and II differ markedly[2]. We present here details of the cavities in II and compare them with those reported earlier for I (ref. 3).

I   II
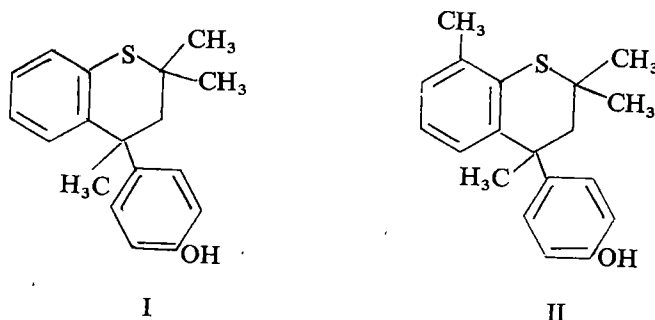
The cyclooctane clathrate of II, which was selected for crystallographic study, is trigonal with a unit cell ($a = 33.629(9)$; $c = 8.239(3)$Å) containing 18 molecules of $C_{19}H_{22}OS$. A host–guest (cyclooctane) ratio of 4.5:1 was measured by ^1H nuclear magnetic resonance for a CDCl$_3$ solution. The space group is either R3 or R$\bar{3}$; (ref. 4) the centrosymmetric space group (R$\bar{3}$) was chosen by analogy to the structure of the clathrates of I (ref. 3) and this choice has been justified by successful analysis. The structure was solved by direct methods using 1,915 independent reflections measured with Mo–K$_α$ radiation on a Hilger–Watts automatic diffractometer, and refined to a current R value of 0.119. All 22 of the hydrogen atoms of the host molecule have been located and allowed for; it has not yet been possible, however, to allow effectively for the guest molecules and this should allow further reduction in R.

Figure 1 illustrates the general similarity of the clathrate host structures of I and II. Both structures contain groups of six molecules the hydroxyl groups of which are linked by
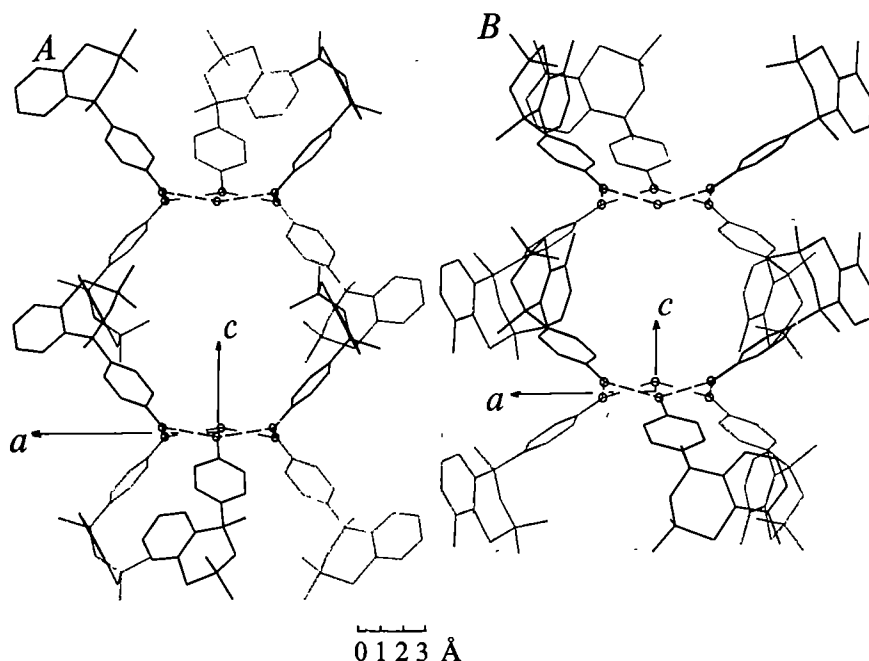
Fig. 1 *A*, Structure of compound I looking on to the *a–c* plane. The guest molecule, 2,5,5-trimethylhex-3-yn-2-ol, is not shown. *B*, Structure of compound II looking on to the *a–c* plane. The (disordered) cyclooctane guest is not shown. In both cases, two host molecules which lie above and below the cavity as viewed in the direction, have been excluded, apart from their hydroxyl oxygen atoms.

0 1 2 3 Å

hydrogen bonds to form a distorted hexagon, alternate molecules lying on opposite sides of its plane. Two such sextets are stacked in each *c* axis repeat and their bulkier parts interlock to form cages. The major differences between the two structures (marked contractions in *c* and in therefore the length of the cavity) are the result of a change in the interlocking pattern which is directly attributable to the introduction of the methyl group in II. Minor changes are: shorter O···O distances in II (2.766(12) compared with 3.035(8) Å) and a more puckered

Fig. 2 Section through the van der Waals' surface of the cavity for I (*A*) and II (*B*).

ring of oxygens (displacements from the mean plane of +0.34 Å (compare 0.22 Å in I)); and the introduction of distortion into the half-chair heterocyclic ring. In I the ring *gem*-dimethyl carbon and the methylene carbon are almost symmetrically displaced (0.38 and 0.34 Å) on opposite sides of the ring, whereas in II the distances are 0.23 and 0.45 Å.

The marked change in cavity shape is most readily appreciated by comparing their van der Waals' surfaces. Thus, Fig. 2*a* shows that the cavity in I has a pronounced waist at $Z = \frac{1}{2}$ which is formed by six *gem*-dimethyl groups protruding into the cage. Figure 2*b*, on the other hand, shows the complete elimination of this waist in II—indeed the central section of this cavity is the widest: the 'hour-glass' surface of I is converted into the 'chinese-lantern' shape of II.

Marked variations in clathrating specificity and in crystallographic data have been reported[5] in a range of specially made chemical variants of the inorganic $M(SCN)_2(R\text{-pyridine})_4$ clathrates (M = Fe, Ni, Co; R = Me, Et, vinyl, and so on). Another deliberate modification of an organic clathrate cage was recently reported[6] by Emert and Breslow who succeeded in introducing a hydrophobic 'floor' into the cavity of β-cyclodextrin, but the alteration in the cavity reported above is much more general and drastic. This report, therefore, provides the first detailed analysis of the changes produced by a piece of planned clathrate engineering.

DAVID D. MacNICOL
ANDREW D. U. HARDY
JOSEPH J. McKENDRICK

Chemistry Department
The University
Glasgow G12 8QQ, UK

1 Hardy, A D. U., MacNicol, D. D., and Wilson, D. R., *Chem. Commun.*, 783–784 (1974).
2 Hardy, A. D. U., McKendrick, J. J, and MacNicol, D. D., *Chem. Commun.*, 972–973 (1974).
3 MacNicol, D. D., and Wilson, F. B., *Chem. Commun*, 786–787 (1971).
4 *International Tables for X-ray Crystallography*, 1 (edit. by Macgillavry, C. H., and Rieck, G. D.; gen ed Lonsdale, K.), 253 (Kynoch, Birmingham, 1965).
5 Bhatnagar, V. M., *Clathrate Compounds*, ch. 4 (Chemical Publishing, New York, 1970).
6 Emert, J., and Breslow, R., *J. Am. chem. Soc.*, 97, 670–672 (1975).

Erratum

In the article "Depths of origin of Kenyan basalts and implications for the Gregory Rift" by G. G. Goles (*Nature*, 255, 391; 1975) the received date is incorrect. It should read Received February 24; accepted April 9, 1975.

matters arising

Superfoetation in fishes and the cost of reproduction

THIBAULT[1] has examined the effects of juvenile mortality on reproduction of normal and superfoetatious females of the family Poeciliidae and concluded that superfoetation conferred no selective advantage in an unpredictable environment in which mortality was catastrophic. A life-table model for the evolution of superfoetation shows this to be wrong.

The net reproductive rate of a population (R_0) summarises the interaction between survivorship and fecundity. In general,

$$R_0 = \sum_{x=0}^{\infty} l_x m_x \qquad (1)$$

where l_x is age-specific survivorship and m_x is age specific fecundity. A consequence of superfoetation is that females produce fewer young more often than usual. For example, *Poecilia reticulata* females 30–35 mm long produce about 24 young every 21 d (ref. 1). They are not superfoetatious. By contrast, *Poeciliopsis lucida* and *P. monacha* are superfoetatious and females 30–35 mm long produce about 11 young every 11 d (ref. 1). A more general comparison of superfoetatious and normal life histories is given in Table 1.

The net reproductive rate of a superfoetatious female producing young twice as often as a normal female is:

$$R_0 = m_x B_s (1 + p_s + p_s^2 + \ldots) \qquad (2)$$

Here, m_x is the number of young produced in interval x, B_s is survivorship to the age of first reproduction for superfoetatious females, and p_s is survivorship to subsequent reproductive intervals. Equation (2) can be simplified[2,3]

$$R_0 = m_x B_s / (1 - p_s) \qquad (3)$$

Similarly, the net reproductive rate of a normal female is:

$$R_0 = n_y B(1 + p^2 + p^4 + \ldots) \qquad (4)$$

Where n_y is brood size, B is survivorship of a normal female to reproductive

age, and p is survivorship to subsequent (x) intervals (Table 1). Equation (4) can be rewritten

$$R_0 = \frac{n_y B}{(1 - p^2)} \qquad (5)$$

We can define the circumstances in which superfoetation and normal patterns of reproduction have equal net reproductive rates, that is, when

$$m_x B_s / (1 - p_s) = n_y B / (1 - p^2) \qquad (6)$$

or when

$$p = \sqrt{\{1 - [(n_y B / m_x B_s)(1 - p_s)]\}} \qquad (7)$$

Whether or not superfoetation is advantageous depends on survivorship to age at first reproduction, subsequent survivorship, and the degree to which brood size is depressed in superfoetatious females. Our example comparing *P. reticulata* with *P. lucida* and *P. monacha* is illustrative. The species are of similar size when they first reproduce. Therefore, we assume equal survival to age at first reproduction. Brood size for the superfoetatious species is half that of the normal female[1], so $m_x = 1$ and $n_y = 2$. Thus equation (7) becomes

$$p = \sqrt{(2p_s - 1)} \qquad (8)$$

The difference in adult survivorship (D) when net reproductive rates are identical for both strategies is defined as

$$D = \sqrt{(2p_s - 1)} - p \qquad (9)$$

D increases as adult survivorship declines (Fig. 1). When the probability of a normal female surviving to produce another brood (11 d in our example[1]) is less than 50%, superfoetation cannot evolve. When survivorship is in excess of 80%, superfoetation results in a higher net reproductive rate if it increases adult survival as little as
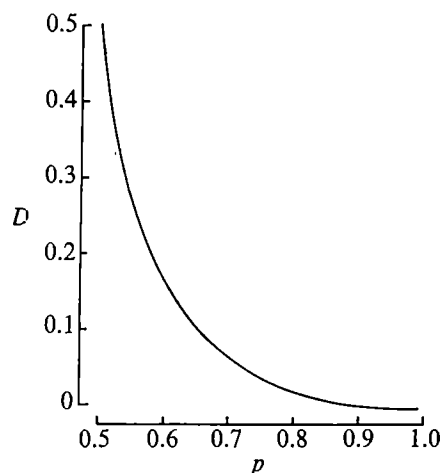
Fig. 1 Relationship between adult survivorship (p) of normal females and the minimum increase in survivorship (D) for superfoetation to be a more advantageous reproductive strategy.

2.5%. *P. lucida* and *P. monacha* occur in unstable environments and invade flood expanded habitats[1] possibly characterised by low competition and predation and, consequently, by high adult survival. As superfoetation will reduce the peak cost of reproduction and further increase adult survival, we interpret it as an adaptation that increases reproduction in transient environments.

JERRY F. DOWNHOWER
LUTHER BROWN

Department of Zoology,
Ohio State University,
Columbus, Ohio 43210

1 Thibault, R., *Nature*, **251**, 138–140 (1974).
2 Mertz, D., *Am. Nat.*, **105**, 437–454 (1971).
3 Goodman, D., *Am. Nat.*, **180**, 247–268 (1974).

THIBAULT REPLIES — Downhower and Brown[1] contend that natural selection will favour fishes with superfoetation over

Table 1	Basic life table for superfoetatious and normal species			
Reproductive event	Superfoetatious		Normal	
	m_x	l_x	n_y	l_x
Birth	0	1.00	0	1.00
↓	↓	↓	↓	↓
First reproduction	m_1	B_s	n_1	B
2	m_2	$B_s p_s^{-1}$	0	$B p^1$
3	m_3	$B_s p_s^{-2}$	n_2	$B p^2$
↓	↓	↓	↓	↓
i	m_i	$B_s p_s^{-i-1}$	n_i	$B p^{i-1}$

fishes without superfoetation in tranisent environments characterised by low levels of predation and competition, and consequently high adult survivorship. Although the mechanics of the model seem sound, its theoretical applicability breaks down on examination of the biology of poeciliid fishes.

In the Rio del Fuerte of north-western Mexico, *Poeciliopsis monacha* inhabits small pools in headwater arroyos carved in solid bedrock. The raging torrents of the rainy season revert in the dry season to congested and isolated pools of a few litres of water. Fishes become compacted, are heavily parasitised and appear emaciated. Aquatic arthropod and reptilian predators are abundant, imposing

and J. Schultz, unpublished). Superfoetation does not seem to be an adaptation solely to transient environments; levels of superfoetation, instead, seem correlated with increased stability and decreased levels of competition. Moreover, approximately 85% of the species of poeciliids lack superfoetation which further attests to the flexibility of the more common and more widely distributed reproductive mechanism.

Further, there is no reason to assume that superfoetation reduces the peak cost of reproduction. Some *Poeciliopsis* have evolved high degrees of maternal contributions to developing embryos with pseudoplacentas (R.T. and J.S., unpublished). The maintenance of several

lomas. Further studies in our laboratory and in others showed that these conclusions were not correct.

The new data showing our misinterpretation were as follows. First, when TdT activity is determined in partially purified extracts from thymus, the highest activity is observed in the presence of oligo (dA) as primer, and of dGTP as substrate. When myeloma extracts were used, no activity was detected with this combination. In our previous study, the TdT activity was determined using $d(pT)_4$ with dATP or dTTP; this system seems to have very low efficiency. Second, further studies of the profile of the activities on the phosphocellulose chromatograms with thymus extracts showed an overlapping, but not an exact correspondence of the peaks obtained with oligo (dA)/dGTP and with $d(pT)_4$/dATP. The $d(pT)_4$/dATP activity was not modified in the thymus after hydrocortisone treatment of mice. It was recently shown that TdT is drastically decreased in these conditions[2,3].

Therefore, the activity we described[1] does not correspond to terminal deoxynucleotidyltransferase, but to another deoxynucleotide-polymerising enzyme, the nature of which remains to be determined.

We thank D. Baltimore, R. MacCaffrey, T. A. Harrison, P. C. Kung and A. E. Silvertone for help in the analysis of myeloma and thymus activities which led us to the correct interpretation of our results.

CLAUDE PENIT
ALAIN PARAF
FRANCOIS CHAPEVILLE

*Institut de Biologie Moléculaire,
Paris, France*

[1] Penit, C., Paraf, A., and Chapeville, F., *Nature*, 249, 755–757 (1974).
[2] Kung, P. C., Silverstone, A. E., McCaffrey, R. P., and Baltimore, D., *J. exp. Med.*, 141, 855–865 (1975).
[3] Bollum, F. J., *Fedn Proc.*, 34, 494 (1975).

Table 1 Calculation of *r* for populations of fishes with and without superfoetation

(10 d interval)	With superfoetation		Without superfoetation	
	m_x	l_x	m_x	l_x
1	0	1.00	0	1.00
↓	↓	↓	↓	↓
8 (First reproduction)	10	$p-n_1$ (0.025)	20	$p-n_1$ (0.025)
9	10	$p-n_2$ (0.025)	0	—
10	10	↓	20	$p-n_3$ (0.025)
11	10		0	—
12	10		20	↓
13	10		0	—
14	10		20	
↓	↓	↓	↓	↓
i	m_i	$p-n_j$ (0.025)		$p-n_j$ (0.025)

n, Integer values from 0 to j.

As p ⟶ 0.8 (high survivorship), *r* ⟶ 0.37   *r* ⟶ 0.41.
As p ⟶ 0.3 (low survivorship), *r* ⟶ 0.32   *r* ⟶ 0.37.

l_x, age-specific survivorship; m_x, age-specific fecundity; *p*, survivorship adjustment factor to determine survivorship at each interval.

---

predation pressure on all size classes of *Poeciliopsis*[2]. Conjecture that these environments are characterised by low levels of intraspecific competition and predation and high adult survivorship, is unfounded.

Downstream from headwater environments, the Fuerte broadens and is somewhat stable with a diversity of lentic and lotic habitats. Inhabited by *P. lucida*, these tributaries are home to an equally diverse array of aquatic predators as well as avian predators. The robustness of fishes in this habitat reflects reduced levels of competition. These tributaries drain into the main stems of the Fuerte with deep and flowing permanent water and stable temperature regimes. Superfoetated *P. prolifica* is found in these environments.

In terms of stability, stream size, and competition levels, the habitat of *P. monacha* lies at one extreme, being transient with competition pronounced. The environment of *P. prolifica*, in contrast, is stable with low levels of competition. The environment of *P. lucida* is intermediate in these characteristics. Predation pressure is variable among the environments. Populations of reproducing *P. monacha* average 1.4–1.5 broods per female developing simultaneously; *P. lucida*, 1.8–2.0; and *P. prolifica* 4–5 (R.T.

broods, each with specific nutritive and metabolic demands, may instead enhance the cost of reproduction.

In extending a hypothetical life table (Table 1) to enable calculation of *r* (the intrinsic rate of increase[3]), fishes without superfoetation have a slight advantage. Regardless of female survivorship, the ability to produce a large number of young in the initial reproductive period may be adaptive in transient environments where *r* selection is operative.

Superfoetation must convey some evolutionary advantages to viviparous fishes; however, the causative selective force, to me, still remains an enigma.

Bowling Green State University.

[1] Downhower, J., and Brown, L., *Nature*, 256, 345 (1975).
[2] Thibault, R., thesis, Univ. Conn (1974).
[3] Birch, L., *J. Anim Ecol.*, 17, 15–26 (1948).

Terminal deoxynucleotidyl-transferase in murine myelomas

WE have published[1] results suggesting the presence of terminal deoxynucleotidyltransferase (TdT) in murine mye-

Double helix of tropomyosin

LONGLEY[1] has suggested that the supercoil pitch length (*P*) of tropomyosin (TM) may be 114 Å. The arguments in favour of this value are based on the assumptions that TM molecules are optimally packed in the tactoids formed using Mg^{2+} and Ca^{2+} (ref. 2) and also that TM presents an axially and azimuthally pseudo-equivalent aspect to consecutive actin molecules.

Although it is known[3,4] that two-stranded ropes may pack optimally when there is a relative axial stagger of *P*/4, it remains to be shown that TM packs in this way in Mg tactoids. Indeed, the Mg

tactoid geometry may simply be explained as follows: the distribution of acidic residues of a TM chain has approximate mirror symmetry about a point $\sim 70\text{Å}$ from the N terminus. The assembly of the tactoids may not be controlled solely from packing considerations, but may be dominated by the bridging effect of Mg^{2+} between acidic moieties of oppositely directed molecules. If TM molecules were optimally packed in the positively and negatively stained tactoids, there is still no *a priori* reason to believe that $P/4$ should be equated to the observed 28 Å axial period.

Even with an axial pseudo-period of about 19.5 residues in acidic and in apolar residues not in series I and II (refs 5 and 6), azimuthally-identical pseudo-equivalent actin–TM interactions could only occur if an actin separation along one strand ($\sim 2 \times 19.5$ residues) corresponds to a multiple of P' (unless the two chains of TM are homologous and in axial register, in which case actin separations could correspond to a multiple of $P'/2$). P' is the supercoil pitch length measured along the helical axis of the supercoil. On packing grounds, it has been suggested that the two chains of TM may have a relative axial stagger of 14 residues[6–8] thus facilitating head-to-tail assembly of TM molecules, but recent evidence has cast doubts on this conclusion[9,10].

Irrespective of the relative chain stagger in TM, the length and flexibility of side chains may enable the first and/or second chain of TM to make pseudo-equivalent interactions with an actin strand even if precise azimuthal relationships are not maintained (see, for example, Hulmes *et al.*[11] for collagen).

Theoretically, the pitch length of α-fibrous proteins may be found directly using a measurement of the axial separation of the equatorial and near equatorial reflections in the X-ray diffraction patterns[12]. Inherent accuracy, however, is usually low as a result of partial overlapping of the relevant data. Also the possibility that axial sampling moves the maxima of the molecular transform cannot be completely eliminated[13]. P has been estimated for α keratin[14] (140–170 Å); paramyosin[4,15] (136–140 Å and 178 Å) and honeybee silk[16] (140 Å), but not for TM. If the head-to-tail assembly of TM molecules in the grooves of the thin filament form a regular supercoil and troponin has an invariable azimuthal relationship with TM and actin, then $nP = 410 \pm 4\text{Å}$ (ref. 17) where n is integral. If $n = 3$, then $P = 137\text{Å}$ and there would be six half-turns of supercoil associated with seven actin molecules. As McLachlan and Stewart (private communication) have also pointed out, TM would then present pseudo-equivalent aspects to all seven actins with which it makes contact, as $3P = 7P'/2 = 410\text{Å}$ where $P'/2 \sim 2 \times 19.5$ residue translations

of an α helix or, equivalently, an actin separation.

The pitch length of an α-fibrous coiled coil cannot be continuously reduced without a concomitant increase in the deformation of bond lengths and angles. The point at which the coiled coil structure reaches a minimum energy state is not known, although it is tempting to speculate that this may occur when P is about 140 Å, a value experimentally noted for several α-fibrous proteins.

D. A. D. PARRY

Department of Chemistry, Biochemistry and Biophysics,
Massey University,
Palmerston North, New Zealand

1 Longley, W., *Nature*, 253, 126–127 (1975).
2 Cohen, C, and Longley, W., *Science*, 152, 794–796 (1966).
3 Rudall, K. M., *Lectures on the Scientific Basis of Medicine*, V, 217–230 (Athlone, London, 1956).
4 Elliott, A., Lowy, J., Parry, D. A. D., and Vibert, P. J., *Nature*, 218, 656–659 (1968).
5 Miller, A., *M.T.P. Reviews of Biochemistry* (edit. by Blaskco, H.), (Butterworths, London, in the press).
6 Parry, D. A. D., *Biochem. biophys. Res. Commun.*, 57, 216–224 (1974).
7 Hodges, R. S., Sodek, J., Smillie, L B., and Jurasek, L, *Cold Spring Harb. Symp. quant. Biol.*, 37, 299–310 (1972).
8 Stone, D., Sodek, J., Johnson, P., and Smillie, L B., *Proc. ninth FEBS meeting, Budapest* (in the press)
9 Johnson, P., and Smillie, L. B., *Biochem. biophys. Res. Commun* (in the press)
10 Stewart, M., *FEBS Lett.*, 53, 5–7 (1975).
11 Hulmes, D. J. S., Miller, A., Parry, D. A. D., Piez, K. A., and Woodhead-Galloway, J., *J molec. Biol.*, 79, 137–148 (1973).
12 Crick, F. H. C., *Acta Cryst.*, 6, 689–697 (1953).
13 Fraser, R D. B., and MacRae, T. P., *Conformation in Fibrous Proteins and Related Synthetic Polypeptides* (Academic, New York and London, 1973).
14 Fraser, R. D. B., *et al.*, *Appl. Polymer Symp.*, no. 18, 65–83 (1971).
15 Cohen, C., and Holmes, K. C., *J molec. Biol*, 6, 423–432 (1963).
16 Atkins, E D. T., *J molec. Biol.*, 24, 139–141 (1967)
17 Cohen, C., Caspar, D L. D., Parry, D. A. D., and Lucas, R. M., *Cold Spring Harb. Symp. quant. Biol.*, 36, 205–216 (1971).

LONGLEY REPLIES—In calculating an example of his model of the coiled coil, Crick[1] started with a straight α helix having 18 units in 5 turns, giving a coiled coil with 126 residues in a pitch of 186 Å. If, instead one starts with the very similar helix of 11 units in 3 turns, the resulting coiled coil has 77 residues in a pitch of 114 Å. (The 11/3 helix is one of the alternatives considered by Pauling and Corey[2] and is the structure automatically formed when an α helix is constructed of CPK molecular models.) Thus, large variations in the pitch of a double helix can result from small changes in the α helix from which it is derived, and there is no reason why the pitch of tropomyosin (TM) should be the same as that of paramyosin, keratin or bee silk. The main resistance to bending the α helix into a tighter coiled coil is in stretching and compressing the H bonds[3]. To bend it into a helix of pitch 114 Å would require 0.1–0.2 kcalorie mol^{-1} which is not large, and would strain the H bonds by only 2 or 3%. The observed spread in H-bond lengths is about 8% (ref. 4).

Now, 77 is an odd number so that each

alternate half-turn of such a double α helix would have a different pattern of side groups from the next. This, together with the 14-residue stagger, could be accommodated in the thin filament of muscle if the pattern of contacts between TM and actin alternated (and perhaps commutated) along the length of the TM molecules. In the 'tactoids' likewise, cross linking (by divalent cations) would be simpler if the chemical repeat were compatible with the helix pitch.

Duke University Medical Center

1 Crick, F. H. C., *Acta Cryst.*, 6, 689–697 (1953).
2 Pauling, L., and Corey, R. B., *Fortschr. Chem. org. NatStoffe*, 11, 180–239 (1954).
3 Crick, F. H. C., *Nature*, 170, 882–883 (1952).
4 Ramakrishnan, C., and Prasad, N., *Int. J. Protein Res.*, 3, 209–231 (1971).

Number counts of γ-ray bursts

STRONG and Klebesadel[1] suggest that most of the 23 γ-ray bursts detected by the Vela satellites are of galactic origin. I question their arguments, which are based on the distribution in galactic latitude and longitude of those bursts for which coordinates have been determined, as well as on the source counts. They admit that the latitude distribution is not significantly galactic. Similarly, the longitude distribution is of low significance. If, *a priori*, the local spiral arm had been predicted to dominate this distribution, then the observations have a chance probability of $\sim 1.5\%$. This probability rises above 6% if the observations in any way suggest the correlation. Further, the location of the event of April 27, 1972 (ref. 2) (which was not used in the reported analysis) does not easily fit into the simple local spiral arm hypothesis.

The source counts as presented give the number of events of time-integrated flux density, S, exceeding some level S_0. As S_0 decreases, the source counts flatten out below the three-halves dependence expected from a uniform source distribution. This seems to indicate a scarcity of weak sources, which is consistent with a galactic origin for the bursts. Such a flattening could perhaps also be caused by the first trigger of the event as well as by the method by which the end of the event is determined. The first trigger effects have been partially eliminated by Strong and Klebesadel and it is evident from their figure caption that the second has been noted, although it is not discussed in the text.

I consider this effect in more detail and assume an abrupt rise and gradual fall-off for the burst represented by:

$$I = 0 \qquad \rbrace t < 0$$
$$I = Ke^{-t/\tau} \qquad \rbrace t \geqslant 0$$

The time-integrated flux S is given by

$$S = K\tau(1 - e^{-t/\tau})$$

where t is the apparent duration of the burst. This will be determined by the detector noise level, which I assume to be constant and to imply a lowest sensitivity of L erg cm^{-2} s^{-1}. Now $t = \tau \ln(K/L)$, therefore $S = (K-L)\tau$.

If the sources are distributed uniformly,

$$N(>K) = CK^{-3/2}$$

and then, $N(>S) = \tau^{-2}C (S/\tau + L)^{-3/2}$

or

$$N(>S) \propto (S + L\tau)^{-3/2} \quad (1)$$

So when $S \gg L\tau$, a three-halves dependence is expected, but when $S \ll L\tau$, $N(>S)$ is independent of S. This is because the events rapidly seem to weaken as K approaches L. The dependence given by equation (1) is of a reasonable form to fit the source counts and implies

$$L\tau \simeq 5 \times 10^{-5} \text{ erg cm}^{-2}$$

τ is not necessarily a constant from event to event and is only representative of the time scale of event decay.

I conclude that the arguments for a galactic origin for γ-ray bursts are by no means sound, and that the distance estimates are extremely uncertain. Number counts of γ-ray bursts, or any other transient phenomena, of time-integrated fluxes less than about $2L\tau$ may merely reflect the average time structure of the events rather than inhomogeneities in source distribution. Many more observations over a much wider range of sensitivity are required if source distances are to be inferred from the number counts. Sensitive γ-ray detectors carried whenever possible in satellite, space-probes or long-duration balloon flights would be invaluable in this respect.

I thank the SRC for support.

A. C. FABIAN

Institute of Astronomy,
Cambridge CB3 OHA, UK

[1] Strong, I. B., and Klebesadel, R. W., *Nature*, 251, 396–397, (1974).
[2] Trombka, J. I., et al., *Astrophys. J. Lett.*, 194, L27–L33 (1974).

STRONG AND KLEBESADEL REPLY—
The arguments used in our letter[1] may be questioned on several grounds, but not those considered by Fabian[2], who makes two principal assumptions. These are the flux as a function of time may be represented adequately by a form having an abrupt rise to maximum followed by exponential decay, and the apparent duration of the bursts is limited by the detector noise level. Given that these were valid we would agree with his analysis. But both assumptions, although seemingly reasonable, are incorrect. The event of April 27, 1972 is an excellent counter-example[3,4]. Our paper did not include this case as it was written before the papers by Metzger et al.[3] and Trombka et al.[4] were published. Further examples may be found in ref. 5.

The actual time profiles of γ-ray bursts are very variable, ranging from single, very intense spikes to complex structures with one or more precursor pulses, a main burst comprising a number of sub-bursts lasting about a second each, and with pronounced substructure, and often a resurgence of weaker, but similar activity a few seconds to a minute later. If a decaying exponential were even an approximately correct model one would expect a strong correlation between S and the apparent duration, t. For values greater than $S = 3 \times 10^{-5}$ erg cm^{-2}, where we have noted that we expect trigger-threshold effects, this is not the case[6]. The Vela detectors can provide reliable values of S well below those of most of the measured events, if we assume the time profiles are similar to the stronger ones. We do not record these events because they fail to trigger the system. In other words, in dealing with low values of S we are trigger-limited rather than limited by the detector noise level.

Although Fabian's detailed argument is therefore not relevant this does not mean that we are still satisfied with our interpretation of the log N–log S plot. We had assumed, like Fabian, that the system would trigger only near the start of an event, and that trigger-threshold effects would appear as an increasing trigger-failure rate with weaker signals. Our threshold figure of 3×10^{-5} erg cm^{-2} meant that we would fail to detect a significant fraction of events with measured $S \lesssim 3 \times 10^{-5}$ erg cm^{-2}. What can happen is that even for $S > 3 \times 10^{-5}$ erg cm^{-2} the instantaneous flux may be low and a trigger, if it occurs at all, can take place after the start of the event. The event[3] of April 27, 1972 illustrates this. The Vela 6A system recorded only the last third of the event, giving 3×10^{-5} erg cm^{-2} instead of $\sim 10^{-4}$ erg cm^{-2}. Putting it simply, we had considered only failures to trigger which gives rise to errors in N. We did not consider errors in S caused by late triggering. The range over which the log N–log S relationship is reliable therefore lies at $S > 10^{-4}$ erg cm^{-2}. This contains too few events to be significant. (We note that the reported values of S from Vela data[6], and those used here, are low by a factor of about two compared with accurate simultaneous measurements made on some other spacecraft, and are probably all underestimated by this amount.)

Turning to the directional distributions, Fabian agrees with our assessment of their significance as being low. They are not therefore to be ignored. The point of our letter was to draw attention to the possibility that the data contained information relevant to the problem of determining the source distances, and not that there was conclusive evidence for a galactic distribution. We also intended to show how future data could be incorporated for this purpose. Work carried
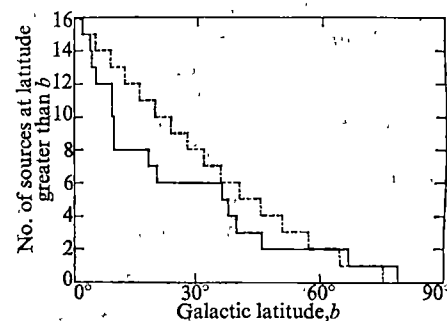
Fig. 1 Integral galactic latitude distribution for 16 gamma-ray burst sources. —, Observed distribution; - - -, isotropic distribution.

out by many people over the past year has provided additional source directions. This has had the effect of largely removing the original anisotropy in longitude, and of further emphasising the preference for low latitudes (Fig. 1). We note that two of the high-latitude sources have large errors ($\sim \pm 15°$) and that one additional source on the Galactic Equator should probably be added.

This is again suggestive of a galactic association, but we accept the possibility that in a few cases we may be witnessing repeats from the same source. If this turns out to be true it is extremely important, but would mean that the distribution of sources as opposed to events would follow more closely the curve for an isotropic distribution.

[1] Strong, I. B., and Klebesadel, R. W., *Nature*, 251, 396–397 (1974).
[2] Fabian, A. C., *Nature*, 256, 347 (1975).
[3] Metzger, A. E., Parker, R. H., Gilman, D., Peterson, L. E., and Trombka, J. I., *Astrophys. J. Lett.*, 194, L19–L25 (1974).
[4] Trombka, J. I., et al., *Astrophys. J. Lett.*, 194, L27–L33 (1974).
[5] Strong, I. B., *Proc. ESLAB Symp. Context and Status of γ-Ray Astronomy* (ESRO, in the press).
[6] Strong, I. B., and Klebesadel, R. A., *Astrophys. J. Lett.*, 188, L1–L3 (1974).
[7] Strong, I. B., Klebesadel, R. A., and Evans, W. D., *Ann. NY Acad. Sci.* (in the press)

reviews

THE value and dangers of drawing analogies between organism and societies have been debated at least since Herbert Spencer; part of their appeal is that they seem to provide a scientific language for talking about society and social organisation, which are prickly subjects of which scientists and doctors usually know little. Dr Salk is open and unashamed about it: "I believe in the usefulness of biological analogies in thinking about men." He calls it "a theoretical–experimental way of thought", to be distinguished from "a 'philosophical speculative way of thought' for dealing with questions in the human realm." He doesn't think much of philosophers, and we hear no more about them, not even those who have studied these problems through the ages. Instead he provides a long series of analogies. Racial intolerance may follow laws similar to immunological intolerance, and "then patterns of possible interest in psychological phenomena, could be observed through quantitative studies in immunology". The phenomena of enzyme induction are held to show something of the environmental forces that are needed to nurture and encourage the fulfilment of our potentialities. He takes the analogies very seriously, but can one really hope that they will, as he suggests, help in adjusting the relationships "between parent and child, teacher and student, lovers,

Biology and man

J. Z. Young

How Like an Angel: Biology and the Nature of Man. By Jonas Salk. Planned and edited by Ruth Nanda Anshen. Pp. 118. (David and Charles: Newton Abbot, London and Vancouver, April 1975.) £3.50.

peers, or between groups or nations"?

The general aim of the book is to show that biological knowledge is increasingly relevant to human affairs, and though this is indeed true perhaps the continual stressing of analogies is not the best way to make the point. Those who actually have to manage social, educational and political affairs are not very likely to pay attention to such generalities, and will ask for more concrete examples. Dr Salk is continually emphasising that we "must" change our ways "What is needed is a change of perspective . . . new values and new ethics are required . . . it will be necessary to establish patterns in the young child that will help him to develop to his fullest." He does not show any understanding that questions as to how these things can be done have occupied men for centuries and are at present being studied intensively.

Those who work in such fields may be annoyed to read that "a beginning must somehow be made". Perhaps more sociology for medical students might be a good start.

In view of Dr Salk's own contributions to infantile paralysis it is natural to look especially carefully at the social analogies he draws with this disease. He indeed believes that there is "a new form of crippling": Man's mind, which has "developed dinosaurian qualities (and) threatens to overpower his body", now apparently "faces uselessness". And our "socially and economically advantaged youths", because of their easy upbringing, are not immunised against this new disorder in the way that they have been immunised against polio. It is not clear whether the conclusion from this analogy is that we should inflict greater hardships upon children of the rich to immunise them against this disease.

It would be unfair to ridicule all of Salk's analogies. He is well up-to-date with studies of the development of the brain and its need for stimulation at appropriate stages of development. Not unexpectedly, he draws an analogy between learning and selective theories of antibody formation. But this is more than an analogy, and it is disappointing that he does not tell us about the evidence that the brain does actually begin with a library of possibilities from which selection is made.   □

PROFESSOR LURIA has produced a very interesting book from the edited transcript of the general biology course he taught at MIT. Its theme is the central place of the genetic programme in biology. In my opinion he succeeds very well in presenting his material (primarily on cell biology) in a logical self-sustaining and interesting manner. Although he provides references to other books he accommodates within his own the essential argument and examples. There are concise appendices on chemical topics such as kinetics, free energy and the structures of biological molecules, and other appendices on discussion topics and examination questions.

The first part of the book (four lecture-chapters) introduces the characteristics of living matter and gives a good account of the cellular components The section on biochemistry

(nine lectures) proceeds from enzymes through energetics and the generation of ATP to the biosynthesis of amino acids, nucleic acids and proteins, but not of carbohydrates and lipids. That on genetics (eight lectures) starts with

---

Lectures in Biology. By S. E. Luria. Pp. xvii+439. (MIT Press : Cambridge, Massachusetts, and London, 1975.) n.p.

Mendel, and deals, in order, with cell cycles and life cycles, bacterial and phage genetics, eukaryotic genetics (almost entirely *Drosophila* and Man) and population genetics and evolution An excellent feature here, and indeed throughout the book, is the way in which Luria demonstrates the value of particular systems in illuminating particular problems without making his treatment seem fragmented. Thus, in the section on developmental biology

(seven lectures) bacterial sporulation, slime moulds, plant gametogenesis and meristems, insects, sponges, *Hydra*, snails, *Amphioxus*, chickens and mice are all used as illustrations. The entry into the discussion of (vertebrate) physiology (eight lectures) is through hormones; there are then chapters on muscle, blood and ionic balance, immunity and neurobiology.

Given the area covered, it is not surprising that the treatment is dogmatic, and that the amount of attention given to how actual experiments were done (and who did them) is generally scant. This is an excellent book for those seeking an exposition of how living systems function. It will be particularly valuable for the more junior university student, physical scientists in search of biology and teachers of biology wanting to keep abreast of cell biology.   **Paul Broda**

Elementary particle theories and phenomena

Particle Interactions at Very High Energies. Edited by David Speiser, Francis Halzen and Jaques Weyers. Part A: Pp. xii+398. $33.60. Part B: Pp. xiii+366. $30.00. (NATO Advanced Study Institutes Series.) (Plenum: London, 1974.)

THESE two volumes cover the main lectures given at the Summer Institute on Elementary Particle Physics held at Louvain in August 1973. Looking back over the years it is probably fair to say that almost every summer school volume that has appeared has contained at least one noteworthy set of lectures, and the present volumes are no exception. Although both books shelter under the umbrella of general elementary particle physics, they are really very different in content and subject matter, and it is, therefore, best to deal with them separately.

Part A is concerned with hadronic interactions at very high energies, and concentrates mainly upon the phenomenological results that have been flowing in such profusion from the CERN Intersecting Storage Rings (ISR) and,

more recently, from the Fermi National Accelerator Laboratory near Chicago. It touches, too, upon the theories and models that have been conjured up to try to interpret the vast amount of data. But if there is a single overwhelming impression left by the reading of this volume it is simply one of amazement and awe; amazement at the absolutely prodigious flow of data from the new machines and awe in the face of the fascinating and complex behaviour of nature at these energies.

There is considerable overlap between several of the lecturers. The most interesting article is Sens', which covers the shadowy area of physics lying between the domain of the high energy physicist and that of the machine engineer: namely the design and structure of the ISR, its beam characteristics, its performance level, its instabilities and their cures, and the connection between the raw measurements and the quantities of physical interest. It is particularly exciting to read of Sens' optimism about the possibility of storing antiprotons in the ISR, using 400-GeV protons from the new CERN Super Proton Synchrotron (SPS) as a source of 25-GeV antiprotons. One awaits with great expectation and some trepidation the results of proton antiproton collisions at ISR energies— many a theory will surely topple.

All three authors concentrate on proton-proton collisions at very high energies, with coverage of elastic reactions, total cross-sections and many body production, the latter in the guise of 'inclusive' reactions. Jacob's treatment of the data is detailed and very wide ranging. Horn applies himself more to "general trends", that is, to the approximate phenomenological rules that seem to summarise the data. Both Jacob and Horn discuss the theoretical background, but neither does so in a sufficiently comprehensive manner to allow the non-expert to follow. Horn does succeed, though, in giving one a taste of the remarkable success enjoyed by Regge theory, in its extension to many body reactions by Mueller, at least in explaining the gross features of the data.

Finally, there are the notes of Halzen (Model Independent Features of Diffraction) which provide a rather pedantic, and sometimes careless, list of theoretical results that can be derived from 'deep' principles, that is, from axiomatic field theory. Some discussion of their relevance, or lack of relevance, to present day experiments is given.

All in all this is an interesting, though not distinguished, collection of notes the value of which is not a little diminished by the outrageous presence of 18 blank pages.

Part B reminds one of how much easier it is to write elegantly on a purely theoretical topic than on a phenomenological one. All the lectures are

clear; arguments are logically developed; examples reinforce one's understanding, and one emerges feeling that one has been subjected to a valuable pedagogical experience.

Gilman (Deep Inelastic Scattering and Final State Hadrons) gives a good summary of the theory of electron-proton and neutrino-proton collisions at large momentum transfer, with a judicious blend of ideas about scaling, partons, the structure of the produced hadrons, and just enough of experiment to motivate the treatment. The article by Weyers (Constituent Quarks and Current Quarks) tackles one of the most tantalising problems in elementary particle theory, namely the relationship between those quarks out of which the hadrons seem to be built, and the quarks in terms of which the weak and electromagnetic currents are simply expressed. There is a useful summary of the pros and cons of the group U(6) as a classification group for the hadrons, and a good pedagogical introduction to the Melosh transformation. Carlitz (Chiral Symmetry and the Hadron Spectrum) repeats much of the material of Weyers, but less adequately. It is hard to justify the duplication. A united effort by the two authors would surely have been more successful

The great mystery about quarks is their non-appearance as identifiable, individual particles in the real world. Models have been blithely advocated in which the quarks are permanently bound inside the hadrons which they constitute. In his article (Permanently Bound Quarks) Mandula presents a fascinating speculative study of a 'field theory' in which the fundamental quanta cannot materialise as free particles. The picture, though a little contrived does expose the basic and somewhat frightening difficulties.

The other principal article in this volume, a major *tour de force* that was originally issued as a CERN Yellow Report, is the evocatively titled "Diagrammar" of 't Hooft and Veltman. Inspired by the difficulties of applying the canonical rules of field theory to gauge theories, they have tried to re-formulate all the essential rules in terms of a diagrammatic calculus (essentially a sophisticated form of Feynman's propagator approach) and to de-emphasise completely both the role of the field and the Lagrangian. Many of the derivations of standard results, for example, of the Cutkosky rules for discontinuities of Feynman diagrams, are far clearer and simpler than in the conventional treatments. The *n*-dimensional regularisation method invented by the authors, is also discussed in some detail. This is a long article and, generally, is very clear, but it leaves one with the feeling that just a little more effort would have ironed out the few irritating sections in which the reasoning is difficult to follow.

E. Leader

Growing crystals

Crystal Growth: Theory and Techniques, vol. 1. Edited by C. H. L. Goodman. Pp. ix + 300. (Plenum : New York and London, 1974.) $33.60.

A FEW years ago essentially only one textbook on crystal growth existed. Today we have a wide choice, particularly as the assembly of a few miscellaneous articles between hard covers frequently masquerades as a textbook. An addition to both categories might be expected to cause only a slight shudder; in fact I found the present volume to be interesting and rewarding.

The articles, ably selected and edited, cover two subjects that have received little previous attention, and two that are both topical and important. The rapid development in crystal growth in recent years has forced redundancy on some of the earlier books on the subject and the appearance of comprehensive reviews aimed at a suitably high level is not only desirable, but perhaps essential if a new generation of crystal growers is to evolve.

The first section, dealing with 'Mechanisms in Vapor Epitaxy of Semiconductors' is adequate but pedestrian. This is compensated for by the excellent content and range of the section entitled 'Principles of Vapour Growth', by E. Kaldis. His *tour-de-force* includes recent work on transport theory, practical techniques and growth data. Kaldis has assembled much useful and interesting information which makes the purchase of the book worthwhile for this section alone.

Travelling solvent techniques are dealt with by two workers who have pioneered these processes and who have achieved some remarkable successes. Although many of the materials dealt with can be grown by other methods, the preparation of large crystals of calcite has demonstrated the possibilities of the technique for a production process. Its wide applicability makes it useful as a research tool for both bulk and thin layer single crystals.

The final article deals with refractory metal crystals. Although specialised, useful information is given on techniques which are possibly not familar to many crystal growers; the section is worth reading by all.

The book has restored my faith in current publications. I await the appearance of further volumes in this series without trepidation.

E. A. D. White

Reproductive aspects

Physiology and Genetics of Reproduction. Parts A and B. (Basic Life Sciences, vol. 4.) Edited by Elsimar M. Coutinho and Fritz Fuchs. Part A: Pp. xxi + 417. Part B: Pp. ix + 454. (Plenum: New York and London, 1974.) $38.50 each.

THESE volumes report the proceedings of an International Symposium on "Physiological and Genetic Aspects of Reproduction", held during December 1973 in Salvador, Brazil, to integrate knowledge of genetics and reproductive biology.

The books contain more than 50 articles covering a wide range of topics. Part A begins with a review on changes in reproductive life-span, the relationship between parity and breast cancer, and the thought-provoking concept that women in 'primitive' societies would normally have been pregnant or lactating and that, therefore, menstruation may be a 'disease' of modern society. The remainder of this volume is subdivided into four sections. The first deals with chromosomal structure and function, sex chromosome activity in germ cells, and chromosomal errors related to gonadal dysgenesis. The second section is concerned with the molecular basis of hormone action, and the third section reviews the control and inhibition of spermatogenesis, the effects of γ rays, and sperm structure and biochemistry. Finally, there are considerations of the role of the central nervous system; releasing hormones; protein synthesis in the egg; follicular growth, ovulation induction, luteinisation and steroidogenesis; and control of the menstrual cycle.

The second volume also contains four sections, the first of which examines structural and biochemical changes in spermatozoa, eggs and oviducts; species specificity in egg-sperm interaction; and inhibition of fertilisation with specific antibodies. Section 2 considers control of contractility in male and female reproductive ducts (ejaculation, tubal transport, uterine movements, and so on), and Section 3 reviews implantation, decidualisation, embryonic development, and chromosomal abnormalities in human abortuses. The final section discusses the control of luteal function, foetal and maternal hormones in pregnancy, uterine activity, and parturition.

The broad scope of these books ensures that they will be of interest to a wide audience, especially as a high proportion of the chapters consist of reviews. The main criticisms apply generally to conference proceedings: some topics are omitted or inadequately covered (oogenesis; contraception) whereas others are repeated in various sections (such as uterine activity). Furthermore, some of the contributions are remarkably similar to others by the same authors published elsewhere. By far the most serious criticism is that the discussions which followed each paper at the symposium have been omitted from the proceedings: such comments are sometimes more useful and thought-provoking than the articles themselves. In spite of these criticisms, however, the books provide a clear insight into a wide variety of fields. **T. G. Baker**
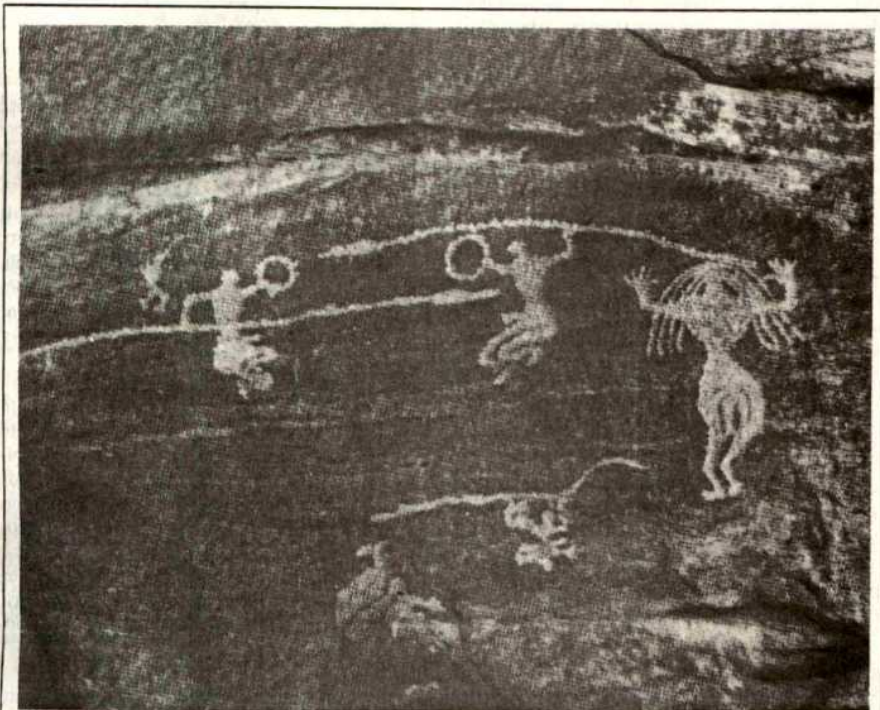
Hunters engraved in rock. From *Rock-Art in Central Arabia.* Vol. 4: *Corpus of the Rock Engravings,* Parts III and IV. Pp. 262. (Institut Orientaliste de l'Université Catholique de Louvain: Louvain-la-Neuve, 1974.) n.p.

Dealing with poisoned patients

The Poisoned Patient : The Role of the Laboratory. (Ciba Foundation Symposium 26, New Series.) Pp. viii+325. (Associated Scientific: Amsterdam, Oxford and New York, 1974.) Dfl. 55; $21.20.

THIS volume comprises 17 papers devoted to various aspects of poisoning; each is accompanied by a very full and frequently thought-provoking discussion.

Almost one third of the papers deal with drug detection and assay techniques, such as gas chromatography linked with mass spectometry, radio immunoassay and associated methodological problems. The controversy over the place of the laboratory in the management of patients who have taken overdoses is covered by Newton and Prescott of Edinburgh who argue spiritedly against Goulding of London and the North American contingent. My impressions are that all of the participants advocate conservative management of the majority of patients and that differences in emphasis of active treatment and demands for drug profile results would disappear if all specialists saw a similar spectrum of patients.

The least well covered aspect in the symposium concerns the role of the laboratory in detecting and preventing drug induced disease. Leach's paper on this subject is mainly concerned with refinement in existing drug assay techniques. The tantalising problem of converting present methods which are mainly concerned with single 'once off' assays into systems capable of dealing with many samples is barely touched on, and no mention is made of the claims of improvement in patient management that have followed the introduction of procedures such as the immunoassay of digoxin. It is estimated that between 5 and 10% of hospital beds are filled by patients suffering from drug-induced diseases. Presumably, that figure indicates the tip of the iceberg with respect to the total quantity of drug-induced ill health.

Dole contributes a stimulating paper on the role of the laboratory in the treatment of narcotic poisoning. In the US it is a federal requirement that the urine of those patients receiving maintenance methadone be tested weekly. That, however, has been found to be positively detrimental to the rehabilitation of patients, as it fosters non-cooperative attitudes.

Repeatedly during the discussions it is claimed that doctors are not sufficiently trained in clinical pharma-cology, so that no matter how sophisticated and accurate are the assay techniques used, the data obtained will be wasted. This book should stimulate interest in clinicians, thus going some way towards overcoming this very real gap in medical education and helping towards the establishment of useful collaboration between clinical and laboratory workers. **Noel Wright**

Biological interface

Biological Interfaces : An Introduction to the Surface and Colloid Science of Biochemical and Biological Systems. By Malcolm N. Jones. Pp. ix+240. (Elsevier Scientific: Amsterdam, Oxford and New York, 1975.) Dfl.34; $13.25.

THERE is no question that some knowledge of surface and colloid chemistry can be a valuable aid to the understanding of a range of biological problems. To date, however, there have been no serious attempts to present in a systematic manner those aspects of surface chemistry which are particularly relevant to biology. Dr Jones has undertaken this quite difficult task and, though he has not produced a definitive work on the subject, he has certainly produced a very readable little volume which should be useful to everyone from undergraduates upwards.

There are eight chapters on topics including surface and interfacial tension, monolayers, micelles, protein–surfactant interactions, electrical double layers, cell surfaces and cell contacts and artificial membranes. In each instance the surface chemical principles are discussed with special regard for biological systems and numerous references are given to the original literature. Herein lies the first of my criticisms, that is, that the references are not, in some cases, as up-to-date as they might be. It may be that this is difficult to avoid in a book on a relatively fast-moving field but, even so, this point should be appreciated. My second concern is that in one or two instances I should like to have seen a more critical presentation of the material. For example, it is not reassuring to find the author putting in a good word for the Pauling–Miller anaesthesia hypothesis, or to see Clements' theory of alveolar stability presented as established fact, or to find that the evidence on the water permeability of bilayers has been inadequately summed up. On most topics, however, the discussion and assessment of the present situation are refreshingly good and, for those starting out in the field, this is a book well worth reading. **D. A. Haydon**

announcements

Appointment

F. R. Jevons has been appointed the first vice-chancellor of Deakin University at Geelong, Victoria, Australia.

Award

A. M. Weinberg has been awarded the first **Heinrich Hertz Prize** for contributions to the science and technology of nuclear energy.

Miscellaneous

Johann–Georg–Zimmermann Prize. Intended to encourage cancer research, the prize (50,000 DM) will be awarded to one or more scientists who have earned special recognition in this field. Part of the prize will be awarded in acknowledgement of outstanding work by young scientists (under 40). For this section of the prize, the subject for 1976 will be 'Surgical and radiological treatment of cancer'. Closing date: January 15, 1976. Information: Freunde der Medizinischen Hochschule Hannover e.V, 3000 Hannover 1, Am Hofen Ufer 6, FRG.

International meetings

September 8–12, **Rhythmic functions in biological systems**, Vienna, Austria (Dr G. Lassmann, c/o Wiener Medizinische Akademie, Alser Strasse 4, 1090 Vienna, Austria).

September 8–12, **Mathematical models for environmental problems**, Southampton, UK (Dr C. A. Brebbia, University of Southampton SO9 5NH, UK).

September 8–12, **Europe from crust to core**, Reading (Mrs D. M. Powell, Local Organising Secretary of MEGS, Department of Geology, The University, Whiteknights, Reading RG6 2AB, UK).

September 8–12, **Applications of ion beams to materials**, Warwick, UK (Professor G. Carter, Department of Electrical Engineering, University of Salford, Salford M5 4WT, Lancashire, UK).

September 8–13, **Carboniferous stratigraphy and geology**, Moscow (Secretary General, Dr Sci Sergei, Viktorovich Meyen, Geological Institute of the USSR Academy of Sciences Carboniferous Organising Committee, Pyzhevsky per 7, Moscow 109017, USSR).

September 9–10, **Proton transfer**, Stirling, UK (Mrs Y. A. Fish, Faraday Division, The Chemical Society, Burlington House, Piccadilly, London W1V 0LQ, UK).

September 9–10, **Morphology and biology of living and fossil reptiles**, London (The Linnean Society of London, Burlington House, Piccadilly, London W1V 0LQ, UK).

September 9–12, **Energy and physics**, Bucharest, Rumania (European Physical Society, PO Box 39, 1213 Petit-Lancy 2, Switzerland).

September 9–12, **Pulsed high beta plasmas**, Abingdon (Mr J. H. C. Maple, Conference Secretary, UKAEA, Culham Laboratory, Abingdon, Oxfordshire ON4 3DB, UK).

September 9–12, **Calcium in biological systems**, Egham, UK (Professor C. J. Duncan, Department of Zoology, The University, PO Box 147, Liverpool L69 3BX, UK).

September 9–13, **Neurosciences**, Munich (Professor D. Ploog, Max-Planck-Institut für Psychiatric, Kraepelin strasse 10, München 40, FRG).

September 10–12, **Reproductive physiology of invertebrates**, Kerala, India (Dr K. G. Adiyodi, Department of Zoology, Calicut University, Kerala 673635, India).

September 10–12, **Thin-layer chromatography and associated techniques**, London, UK (Laboratory News Europe, 78 Wigmore Street, London W1, UK).

September 10–17, **Virology**, Madrid (Dr. R. Najera, Centro Nacional de Virologia y Ecologia, Sanitarias, Majadahonda, Madrid, Spain).

September 11–12, **Computers in laboratory use**, London (The Meetings Officer, Institute of Physics, 47 Belgrave Square, London SW1X 8QX, UK).

September 11–18, **Neurology**, Amsterdam (Preliminary Congress Secretariat, University Clinic of Neurology, Paviljoen 2, Wilhelmina Gasthuis, Amsterdam, The Netherlands).

September 12–21, **Surface membrane receptors**, Bellagio, Italy (Dr R. A. Bradshaw, Director, Department of Biological Chemistry, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, Missouri 63110).

September 13–17, **Transplutonium**, Baden-Baden, German Federal Republic (Professor W. Muller, European Institute of Transuranium Elements, Kernforschungzentrum Karlsruhe, Pöstfach 22600, 7500 Karlsruhe, German Federal Republic).

September 14–19, **Environmental monitoring**, Las Vegas, Nevada (E. A. Schuck, National Environmental Research Center, US Environmental Protection Agency, PO Box 15027, Las Vegas, Nevada 89114).

September 15–16, **Prostaglandin**, Halle/Saale, DDR (Professor W. Forster, Institut fur Pharmakologie und Toxikologie der Universitat, 402 Halle/Saale, Leninallee 4, DDR).

September 15–19, **Beam-foil spectro-scopy**, Gatlinburg (Professor W. W. Havens, Jr, American Physical Society, 335 East 45th Street, New York, New York 10017).

September 15–19, **Liquid scintillation counting**, Bath, UK (Mr M. A. Crook, The Society for Analytical Chemistry, 9–10 Savile Row, London W1X 1AF, UK).

September 15–20, **Cnidarian fossils**, Paris (Dr J. P. Chevalier, Institut de Paleontologie, 8 rue de Buffon, 75005 Paris, France).

September 16–17, **Fluidised combustion**, London (Dr P. Eisenklam, Department of Chemical Engineering, Imperial College, London SW7 2AZ, UK).

September 16–18, **Neurohypophysial hormones**, Liblice, Czechoslovakia (Dr M. Zaoral, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Praha 6, Felmingovonam 2, Czechoslovakia).

September 16–19, **Optical fibre communications**, London (The Conference Department, The Institution of Electrical Engineers, Savoy Place, London WC2R 0BL, UK).

September 16–19, **Offshore Europe**, Aberdeen (Exhibition Administration Limited, 4 Pine Walk, Surbiton, Surrey, UK).

September 17–19, **Growth hormone and related peptides**, Siena, Italy (Mrs M. L. Pecile, Department of Pharmacology, School of Medicine, University of Milano (SM-UM), 32 Via Vanvitelli, 120129 Milano, Italy).

September 17–19, **Clinical biochemistry**, Bratislava (R. Muller, Congress Office, Slovak Medical Society, Mickiewiczova 18, 88322 Bratislava, Czechoslovakia).

September 17–20, **Kinetics and biopolymers**, Jena, DDR (Professor H. Berg, Zentralinstitut fur Mikrobiologie und Experimentelle Therapie der AdW, 69 Jena, Beuthenbergstrasse 11, DDR).

September 21–24, **Electromyography**, Rochester, Minnesota (W. C. Wiederholt, Secretary Treasurer, 7010 Via Valverde, La Jolla, California 92037).

September 21–25, **Immunology of reproduction**, Varna, Bulgaria (Third Symposium on Immunology of Reproduction, 73 Lenin Avenue, Sofia 13, Bulgaria).

Reports and publications

Great Britain

Wildlife Conservation in Charnwood Forest—Report by a Working Party. Pp. 51. (Huntingdon: East Midlands Regional Office, Nature Conservancy Council, Monks Wood Experimental Station, Abbots Ripton, 1975.) £1.50   [205

Proceedings of the Conference on Animal Feeds of Tropical and Subtropical Origin, held at the London School of Pharmacy, Brunswick Square, London, WC1N 1AX, 1st–5th April 1974. Pp. 347. (London: Tropical Products Institute, 56/62 Grays Inn Road, WC1, 1975.) £4.05.   [205

Flora of Tropical East Africa. Edited by R. M. Polhill. Melastomataceae. By Dr. G. E. Wickens. Pp. 95. £1. Dioscoreeceae. By E. Milne-Redhead. Pp. 26. 35p. (London: Crown Agents for Overseas Governments and Administrations, 1975.)   [215

Bulletin of the British Museum (Natural History). Geology. Vol. 25, No. 5: A Revision of Sahni's Types of the Brachiopod Subfamily Carneithyridinae. By U. Asgaard. Pp. 317–365 + 8 plates. £4.50. Zoology. Vol. 28, No. 1: A Guide to the Species of the Genus *Euplotes* (Hypotrichida, Ciliatea). By C. R. Curds. Pp. 1–61. £3.80. Vol. 28, No. 2: Catalogue of the Types of Terrestrial Isopods (Oniscoidea) in the Collections of the British Museum (Natural History). II. Oniscoidea, Excluding Pseudotracheata. By J. P. Ellis and R. J. Lincoln. Pp. 63–100. £2.55. Vol. 28, No. 3: The Larval Development of *Carcinus maenas* (L.) and *C. Mediterraneus* Czerniavsky (Crustacea, Brachyura Portunidae) Reared in the Laboratory. By A. L. Rice and R. W. Ingle. Pp. 101–119 + 1 plate. £1.35. Vol. 28, No. 4: A Comparative Study of the Larval Morphology of the British Portunid Crabs *Macropipus puber* (L.) and *M. Holsatus* (Fabricius), with a Discussion of Generic and Sub-Familial Larval Characters within the Portunidae. By A. L. Rice and R. W. Ingle. Pp. 121–151. £1.90. (London: British Museum (Natural History), 1975.)   [225

Department of Industry. Technology and the Environment. (Reports from Scientific Counsellors Overseas, No. 8.) Pp. 34. (London: Department of Industry, Abel House, John Islip Street, SW1, 1975).   [225,

Cotton Research Corporation. Annual Report for 1974. Pp. 19. (London: Cotton Research Corporation, 1975.)   [275

Proceedings of the Royal Irish Academy. Vol. 75, Section A, No. 9: Thermodynamic Influences on the Propagation of Electromagnetic Shock Waves. By M. J. McCarthy and P. M. O'Leary. Pp. 85–96. 40p. Vol. 75, Section B. No. 12: Studies on the Synthesis of Disaccharides—I. Synthesis of Gentiobiose and 6.0-β-D-Galactopyranosyl-D-Galactise. By Elizabeth E. Lee, Angela Hartigan and P. S. O'Colla. Pp. 267–273. 17p. No. 13: Irish Botany in the Seventeenth Century. By M. E. Mitchell. Pp. 275–284. 26p. No. 14: The Food of the Cormorant *Phalacrocorax carbo* at Some Breeding Colonies in Ireland. By B. West, D. Cabot, and M. Greer-Walker. Pp. 285–304. 50p. (Dublin: Roya Irish Academy, 1975.)   [275

List of University Institutions in the Commonwealth. Pp. 32. (London: The Association of Commonwealth Universities, 1975.)   [275

University of Cambridge. Report of the Head of the Department of Engineering for the Academical Year 1973–74. Pp. 10. (Cambridge: The University, 1975.).   [285

Other countries

Beitrage Zur Geobotansischen Landesaufname Der Schweiz. Heft 55. Vegetationsentwicklung und Waldgrenzschwankungen des Spat-und Postglazials im Oberhalbstein (Graubunden/Schweitz) mit besonderer Berucksichtigung der Fichteneinwandering. Von Christian Heitz. Pp. 63. (4 Diagrams.) (Bern: Verlag Hans Huber, 1975.) Sw. Fr. 30.   [75

Doctors and Healers. By Alexander Dorozynski. Pp. 63. (Ottawa: International Development Research Centre, 1975.)   [85

Republic of the Sudan, Ministry of Agriculture, Agricultural Research Division. 1967–1968 Annual Report of the Hudeiba Research Station. Pp. 78. (Wadi Medani: Agricultural Research Corporation.) n.p. [95

Geophysical Institute, University of Alaska. Annual Report, 1973/1974. Pp. 169. (Fairbanks, Alaska: Geophysical Institute, University of Alaska, 1975.)   [125

Methodological Guidelines for Social Assessment of Technology. Pp. 146. (Paris: OECD; London: HMSO, 1975.) 22 francs; £2.20; $5.50.   [125

Canada: Department of Energy, Mines and Resources. Geological Survey of Canada. Bulletin 228: Quaternary Geology and Geomorphology of Assiniboine and Qu'appelle Valleys of Manitoba and Saskatchewan. By R. W. Klassen. Pp. 61. $4. Bulletin 243: The Jurassic Faunas of the Canadian Arctic. By Hans Frebold. Pp. 34 (5 plates). $4. Paper 74–16: Jurassic and Lower Cretaceous Paleogeography and Depositional Tectonics of Porcupine Plateau, Adjacent Areas of Northern Yukon and Those of Mackenzie District. By J. A. Jeletzky. Pp. 52. $2.50. Paper 74–63: Computer-Based Systems for Geological Field Data. (An International State-of-the-Art Review for 1973 conducted by COGEODATA, International Union of Geological Sciences, in collaboration with the Division of Earth and Environmental Sciences, UNESCO, and the Canadian Centre for Geoscience Data.) By W. W. Hutchison. Pp. 100. (Ottawa: Information Canada, 1975.) $4.80.   [155

Bulletin of the Fisheries Research Board of Canada. No. 190: Hydrodynamics and Energetics of Fish Propulsion. By P. W. Webb. Pp. x + 158. $6. No. 192: Catalogue and Synopsis of *Caligus*, a Genus of Copepoda (Crustacea) Parasitic on Fishes. By L. Largoli,s Z. Kabata and R. R. Parker. Pp. vi + 117. $6. (Ottawa: Information Canada, 1975.)   [155

United States Department of the Interior: Geological Survey. Water-Supply Paper 1999-N: Quality of the Ground Water in Basalt of the Columbia River Group, Washington, Oregon, and Idaho. By R. C. Newcomb. Pp. iv + 71. (Washington, DC: Government Printing Office, 1972.) 75 cents.   [195

Smithsonian Contributions to Zoology, No. 178: Revision of the Cypridinacea of the Gulf of Naples (Ostracoda). By Louis S. Kornicker. Pp. 64. $1.95 No. 179: West African Myodocopid Ostracoda (Cylindroleberididae). By Louis S. Kornicker and Francisca Elena Caraion. Pp. iii + 78. $1.75. (Washington, DC: Smithsonian Institution Press, 1974. For sale by US Government Printing Office.)   [195

The Right to Conduct Nuclear Explosions: Political Aspects and Policy Proposals. (Stockholm Paper No. 6.) Pp. 24. (Stockholm: Stockholm International Peace Research Institute, 1975.)   [195

Chemical Disarmament: New Weapons for Old (Stockholm International Peace Research Institute Monograph.) Pp. viii + 151. (Stockholm: SIPRI and Almqvist and Wiksell International; New York: Humanities Press, 1975.)   [195

Safeguards Against Nuclear Proliferation. (A SIPRI Monographs.) Pp. viii + 114. (Stockholm: Almqvist and Wiksell International: Cambridge, Mass. and London: The MIT Press, 1975.) Sw. kr. 49.   [195

CERN—European Organization for Nuclear Research. CERN 75–3: Radiation and Fire Resistance of Cable-Insulating Materials Used in Accelerator Engineering. By H. Schinbacher and M. H. Van de Voorde. Pp. 15. (Geneva: CERN, 1975.)   [195

Annalen der Meteorologie (Neue Folge), Nr. 9, Die Meteorologen-Tagung in Bad Hombirg v.d.H.: vom 27. bis 29. Marz 1974. Pp. 161. Berichte des Deutschen Wetterdienstes. Nr. 134 (Band 17): Entwicklung eines Sky-Scanners zur Schnellen Vermessung der Raumlichen Verteilung Spektraler Himmelsstrahldichten. Von Klaus Deene. Pp. 37. Nr. 135 (Vand 17): Zwei Wetterkatastrophen des Jahres 1972—Der Niedersachsen-Orkan und das Gewitterunwetter von Stuttgart. Von A. Cappel und P. Emmrich. Pp. 84. (Offenbach A.M.: Selbstverlag des Deutschen Wetterdienstes, 1974 und 1975.)   [205

Records of the Australian Museum, Vol. 29, No. 11: Contributions to the Knowledge of the Alpheid Shrimp of the Pacific Ocean. Part XVIII: A New Species of the Genus *Alpheus* from the Mouth of the Sepik River, New Guinea. By Albert H. and Dora M. Vanner. Pp. 261–266. (Sydney: The Australian Museum, 1975.) 50 cents.   [205

United States Department of the Interior: Geological Survey. Water-Supply Paper 1880-B: Floods of September-October 1967 in South Texas and Northeastern Mexico. By Elmer E. Schroeder, R. U. Grozier, D. C. Hahl and A. E. Hulme. Pp. vi + 111. (Washington, DC: Government Printing Office, 1974.) $3.55. [215

Smithsonian Contributions to Zoology, No. 184: The Genus *Coptocarpus* Chaudoir of the Australian Region with Notes on Related African Species (Coleoptera : Carabidae : Oodini.) By Terry L. Erwin. Pp. 25. (Washington, DC: Smithsonian Institution Press, 1974. For sale by US Government Printing Office.) 85 cents.   [215

Psychopharmacology Communications, Vol. 1, No. 1, 1975. Pp. 1–115. Subscription rate for Vol. 1 (1975) containing six issues $35 (prepaid). Postage outside the United States is $6.39 per volume. (New York: Marcel Dekker Journals, 1975.)   [225

A National Statement by the Faculties of Agriculture and Veterinary Medicine at Canadian Universities. Pp. 22. (Ottawa: Science Council of Canada, 1975.) [225

Australian Academy of Science. Science and Industry Forum Report No. 7: PhD Education in Australia—The Making of Professional Scientists. Pp. 212. (Canberra, ACT: Australian Academy of Science, 1974.)   [275

The Epidemiology of Cancer in Papua New Guinea. Pp. 184. (Port Moresby, Papua: Department of Public Health, 1974.) $10.   [275

Official Records of the World Health Organization No. 221: The Work of WHO 1974. (Annual Report of the Director-General to the World Health Assembly and to the United Nations.) Pp. xviii + 343. (Geneva: WHO: London: HMSO, 1975.) Sw. fr. 18.   [275

Environment Canada. Fisheries and Marine Service. Technical Report No. 521: Eggs, Larvae and Juveniles of Fishes from Plankton Collections in the Gulf of St. Lawrence During 1969. By A. C. Kohler, D. J. Faber and N. J. McFarlane. Pp. 154. No. 534: Underwater Biotelemetry, an Annotated Bibliography. By Aivars B. Stasko. Pp. 29. (St. Andrews, New Brunswick: Research and Development Directorate, Biological Station, 1975.)   [285

Bulletin of the American Museum of Natural History, Vol. 155, Article 2: A Taxonomic Study of African Allodapine Bees (Hymenoptera, Anthophoridae, Ceratinini). By Charles D. Michener. Pp. 67–240. (New York: American Museum of Natural History, 1975.) $8.55.   [295

Smithsonian Contributions to Zoology, No. 181: The Costate Species of *Colaspis* in the United States (Coleoptera : Chrysomelidae). By Doris H. Blake. Pp. iii + 24. (Washington, DC: Smithsonian Institution Press 1974. For sale by US Government Printing Office.) 65 cents.   [295

Smithsonian Contributions to Zoology, No. 186: A Revision of the South American Fishes of the Genus *Nannostomus* Gunther (Family Lebiasinidae). By Stanley H. Weitzman and J. Stanley Cobb. Pp. iii + 36. (Washington, DC: Smithsonian Institution Press, 1975. For sale by US Government Printing Office.) $1.10.   [305

United States Department of the Interior: Geological Survey. Professional Paper 726-C: Gravity and Magnetic Features as Related to Geology in the Leadville 3-Minute Quadrangle, Colorado. By Ogden Tweto and J. E. Case. Pp. iii + 31 + plate 1. (Washington, DC: Government Printing Office, 1972.)   [305

Oh, New Delhi; oh, Geneva

HAS the presence of the World Health Organisation (WHO) in India at the Research Unit on Genetic Control of Mosquitoes, New Delhi been a serious attempt to raise the health standards of the Indian people—or has WHO been, perhaps unwittingly, a vehicle for the United States to extend its knowledge of biological warfare procedures? That question has led to some appalling misunderstandings on the international science scene, the publication of a report by the Indian government strongly condemnatory of the operation and now the entire withdrawal of the WHO from the unit. There are some harsh lessons for all concerned in the extraordinary, and little publicised, events of the last year.

The programme

Three species of mosquitoes were earmarked for attention when the unit was established in 1970; *Culex fatigans*, *Aedes aegypti* and *Anopheles stephensi*. *C. fatigans* was the first to be studied in detail; it is the most ubiquitous nuisance mosquito and the vector of filariasis in many parts of India. *A. aegypti* is less widely distributed; it is virtually absent in villages and not common in most towns. It is the only proved natural vector of urban yellow fever; it is also a vector of dengue and chikungunya. *A. stephensi* is the principal vector of malaria.

It is widely accepted that since a mosquito control programme must take note of a much wider ecosystem than simply that of the mosquito in question, it is bound to contain elements of many different control techniques. Genetic control, the most recently promising technique, started to be widely discussed in India in the early 1960s.

Genetic control may be by one of several methods, amongst which are:
● sterile male techniques; males are sterilised by radiation or chemical treatment;
● chromosomal translocations: the normal chromosome arrangement can be broken by radiation or chemical treatment—the abnormal rearrangement or translocation does not normally cause sterility, but the offspring carry the translocations and are more likely to be sterile.
● cytoplasmic incompatibility: some strains of a given species show mutual incompatibility, and the eggs of such matings are infertile; the introduction of an alien strain thus promotes suppression of a population.

First moves to establish a joint WHO/Indian Council of Medical Research (ICMR) unit to study genetic control were made in 1969. An agreement between the WHO and the Government of India was signed in June 1969 and authorised research on the three species mentioned above.

Progress would be reviewed by the representatives of ICMR, WHO, the US Public Health Service (USPHS) and the (Indian) National Institute of Communicable Diseases (NICD). PL-480 funds from the US government would be made available, and in addition WHO would support a Project Leader, 2 other professional staff and underwrite various other expenses. The agreement was to run for six years in the first instance. Staff levels early in 1975 were: 2 foreign scientists (WHO staff), 13 Indian scientists, and 139 other Indian staff.

In the first four years, work was concentrated on *C. fatigans* partly because there had already been some success in genetic control of this species by chemosterilisation in the United States and by cytoplasmic incompatibility in Burma. Twelve villages in the vicinity of Delhi were singled out for attention, and it was hoped that they could be treated as islands with trivial migration over the kilometre or two that typically separates villages. A variety of sterilisation techniques were employed and mosquitoes were released either as pupae or adults.

Unfortunately it was soon discovered that breeding grounds for *C. fatigans* were not confined to drains and pits in the villages but were fairly uniformly distributed over the countryside in irrigation wells. Further, *C. fatigans* proved to be a good traveller; sometimes as much as 85% of the adult population of a village were immigrants from up to ten kilometres away.

Thus the field experiments, although they showed maximum sterilities on egg rafts of well over 50 per cent were somewhat disappointing.

First criticism

All was not well, however, in the public understanding of these experiments. On the one hand, it was neces-

sary to work hard at the local level to convince villagers that the release of vast numbers of mosquitoes and the associated operations might ultimately be beneficial. On the other there was evidence of some hostility to the programme at a scientific level when the *National Herald* for February 11, 1972 published an article 'Science or Neo-imperialism' by 'A Scientific Worker'. The writer declared that international cooperation in science could often degenerate into a donor-recipient relationship with little opportunity for the recipient to evaluate and influence the work, especially if the recipient nation's scientific elite "is so brain-washed that they are at ease only in the donor nation's social outlook and science programmes". This was to be exemplified by the mosquito programme, run for the benefit only of the US Department of Agriculture (USDA) which could use India as a test-bed for chemosterilisation experiments too dangerous to be permitted in the United States. For, the argument ran, the unit should have tried the three sterilisation methods of irradiation, incompatibility and chemicals and chosen the most appropriate, but it had not purchased a cobalt-60 source necessary for irradiation, nor had it pursued much research on incompatibility. The emphasis on chemosterilisation was inevitable, continued the article, since the first two project leaders were from the USDA with long experience of and a continuing enthusiasm for chemical methods.

This was slightly odd, as two of the five field experiments already performed had used irradiation techniques and a large experiment just about to be performed was to use the incompatibility technique.

The newspaper report went on to describe the chemical used (thiotepa) as a modification of mustard gas, producing 'mutations, cancer and foetal deformities in experimental animals." In one experiment "50,000 mosquito pupae, dipped in the dangerous chemical for 3 h and washed with water, were placed in all the wells of a small village. No one bothered how much residue of the dangerous chemical absorbed or sticking to the insects could directly pollute the drinking water." This is at variance with the unit's own version of these experi-

ments. They claim that drinking water wells were never used, only between six and ten disused irrigation wells; cans of the sterilised pupae were floated in the experiment in question, and in later experiments suspended above the water level. They further claim that chromatographic analysis showed an extremely rapid breakdown of thiotepa residues in the body of the mosquito (half-life, 6 hours).

The unit's recently issued version of these experiments should not go entirely unchallenged. Dr R. Pal who was with the unit during the experiments and is now at WHO headquarters wrote in a review book 'The Use of Genetics in Insect Control' of the same experiment described in the *National Herald*, 'Initially mortality of the released pupae was heavy due to release into the village well water' (so containers of laboratory water were used). Nonetheless, the implication behind the article was that this was the way things were always being done whereas it is clear that the unit's techniques evolved quite rapidly; indeed by the beginning of 1972 pupae were to be used no more.

Even though the article could be faulted as erroneous scaremongering with a touch of anti-Americanism added in, it carried one clear message—that someone close to, perhaps even within the unit was seriously dissatisfied. No doubt it put the unit on its toes to ensure that its future operations could not be faulted; this was to have unfortunate consequences.

More recent work

Work on *Aedes aegypti* at the unit started much later and never reached anything like the intensity of work on *C fatigans*. After two preliminary studies in Delhi in which laboratory strains were successfully incorporated into the normal population, a more extensive study was done in Sonepat City in 1973 along the same lines. There had been considerable optimism that *A. aegypti* would yield definitive results more quickly on the feasibility of genetic control methods since the densities of the mosquito are generally lower than those of *C. fatigans*, and *A. aegypti* has more limited ability to migrate. Genetic control experiments were planned for February 1975 but, as we shall see, were not caried out.

Work on *Anopheles stephensi* was even less advanced; it was only in 1973 that a scientist was hired specifically to work on *A. stephensi*, but the intentions since then have been to increase emphasis on the species in the unit. First field trials were planned for 1975.

New criticism

Work at the unit was struck a major blow by the appearance of a newspaper article on July 29, 1974 entitled 'WHO works for US secret research in India' and written by Dr K. S. Jayaraman, Science Correspondent of the Press Trust of India. The report was fiercely critical of three operations; the other two were the Bombay Natural History Society's bird migration study and a malaria eradication programme in Jodhpur. Dr Jayaraman wrote of tight secrecy in the mosquito project. Some experts, he claimed, believed data could be useful in germ warfare, or that India was being used as a guinea pig "for chemicals or methods not permitted in sponsoring countries." The experiments "appear to have no relevance to malaria and filariasis." Instead the unit was collecting data on *A. aegypti*, a vector of yellow fever—of which "there had not been a single case in India for ages." *Aedes aegypti* could be very useful in transmitting viruses "because its eggs (unlike those of other mosquitoes) can be dried, put on a piece of paper in an envelope and mailed to any part of the country where they can hatch." Yellow fever was believed to be absent in India because of natural cross protection from another virus. "Experts ask if genetic mosquito experiments would affect or remove this natural cross protection. If it does, yellow fever would strike India."

The story had evolved in an interesting way. When Dr Jayaraman first started to enquire about the unit in April 1973 he met with some reluctance from its then project leader to speak in other than generalities, owing to the unfavourable publicity received in 1972. A new project leader, Dr G. Brooks, said in December 1973 that he would get clearance from WHO in Geneva for a more detailed briefing, but clearance never came. Meanwhile the West German embassy in Delhi published, in February 1974, a magazine *German News* in which were printed the views of Professor H. Laven, an insect geneticist from Mainz and regular consultant to the unit. Laven who had worked for many years on incompatibility techniques, was critical of the chemosterilisation experiments which, he declared, had left the mosquito population unaffected. Jayaraman confronted Brooks with Laven's statement and as a result the Press Trust of India carried both Laven's article and Brooks' short rejoinder. But Brooks was still unable to get WHO clearance for more detailed information to be given to Jayaraman.

Jayaraman then contacted Dr R. Pal the Indian malariologist who was responsible for the unit's programme at WHO's headquarters. Pal told Jayaraman that he could give no more details of the operation as it was 'sensitive to the Indian press', presumably as a result of the earlier *National Herald* article. At about this time, Jayaraman alleges, he was indirectly sounded out for a job as an information officer at WHO headquarters.

Response

There were two major consequences of the publication of Jayaraman's article—scientists rapidly reviewed the programme, and a rather slower political investigation was set up. The scientific and administrative review was conducted under the auspices of ICMR. There was obvious concern that the WHO/ICMR agreement needed some tidying up to strengthen Indian involvement in the management of the unit. In particular the consultative role of the Indian counterpart originally provided for in the agreement to assist the project leader needed to be taken more seriously. The scientific review committee, consisting of twenty-one members, could find little wrong with the unit's operations, however, and congratulated the unit on its achievements. Its only recommendation of substance was that an independent monitoring body should be established to oversee future releases of genetically manipulated mosquitoes.

The perspicacious reader of this complicated saga will recognise at this stage all the ingredients to ensure that the political investigation would be explosive:
● a complex scientific investigation with, like all such, its failures and blind alleys as well as its successes
● genuine and sincere disagreement amongst scientists about the correct procedures
● a journalist kept at arms length
● an agency in Geneva suspicious of the Indian press
● US involvement both through money and personnel
● a scientific review that seemed to close ranks.

The consequence was almost inevitable.

It was helped by the way that the Public Accounts Committee went about its business; it indulged in what the Americans would call a fishing expedition. Everything that could possibly be found against the project or those involved in it was brought out —from a contravention of the Registration of Books Act, in not printing the publisher's name on leaflets distributed to villagers, to doubts on Dr Brooks' abilities as project leader (he had only obtained his PhD in 197 (though by then he had worked as a public health entomologist for 1 years).

In January 1975 the committee heard representatives of ICMR and NICI and of the Ministries of Health and Family Planning, Defence and Agriculture, of whom technical and administrative questions were asked. The com

mittee then examined Dr T. Rama-chandra Rao, a distinguished entomo-logist who had worked closely with the unit since his retirement as director of the Virus Research Centre, Poona. Finally, the committee in March 1975 took evidence from Dr Jayaraman and his Editor-in-Chief Mr C. Raghavan. The report was published in late April.

Were they fair?

It is perhaps not insignificant that nowhere in the report is the unit given its correct title by the committee or the journalists—it is always referred to as the Genetic Control of Mosqui-toes Unit, the word 'Research' some-how being lost; perhaps also lost at the same time was some understanding of the complex, frustrating, and often illogical nature of research. It is per-haps also not insignificant that Mr J. Bosu, chairman of the committee had already sufficiently made up his own mind, even before hearing the journa-lists (who were the only witnesses to level accusations of biological warfare), to write to Mrs Gandhi in late January accusing the US of using the pro-gramme, first, to carry out harmful experiments in India which would not be permitted in the US, second, to make preparations in case the US ever wished to wage chemical, bacterio-logical and virus warfare against India, and third, to prepare for such warfare using India as a base.

It also seems unfortunate that the committee should have delayed hearing the journalists who were presenting, as it were, the case for the prosecution until after they had heard all other witnesses. Dr Jayaraman and Mr Rag-havan presented an imposing and detailed case but no-one was then asked to review this case scientifically.

One is forced to the inevitable con-clusion in reading the committee's report and particularly its recommen-dations that its mind was clearly made up that the unit was involved in nefari-ous activities, and that the testimony of the journalists was only needed to provide confirmation. But the journa-lists' evidence needed the most detailed point-by-point investigation. For ex-ample although the journalists took a swipe at almost everything in sight, their central argument could probably be summarised as that the unit was devoting an unreasonable amount of its effort to *A. aegypti* which could be used as a vector of yellow fever. For instance, Mr Raghavan said 'An analy-sis of GCMU's (*sic*) activities in the last five years suggests that GCMU is primarily interested in the collection of data on the ecology and dispersal of Indian mosquitoes, particularly *Aedes aegypti* which is a vector of yellow fever.' The remark necessarily went unchallenged and in its conclu-sions the committee spoke of the unit's

'preoccupation' with *A. aegypti*. But if the committee had even bothered to do a simple thing like count up the num-ber of papers published by the unit in its past five years, it would have seen the *C. fatigans* papers outnumbered *A. aegypti* papers by a ratio of four to one.

Likewise assertions that India's im-munity to yellow fever might be weak-ened by experiments eliminating *A. aegypti* were not confronted with elementary arguments such as that *A. aegypti* already does not exist at all in many Indian cities and nearly all villages—indeed it was eliminated from Poona in 1953 without any incidence of yellow fever.

Biological warfare

Since the committee preferred the journalists' views to those of Dr Rao and the Ministries on all matters of dispute it was easy for it to follow them in the step from believing that the unit was preoccupied with *A. aegypti* to believing that preoccupation with *A. aegypti* meant preparation for biological warfare. It is not clear that the committee believed that any of the foreign scientists working at the unit were witting tools of the US Depart-ment of Defense, but it was certainly convinced that evil purposes lay behind it all. After all, the committee was told that the US Public Health Service (which releases PL-480 funds to WHO) had been known to receive money from the US Biological Warfare Research Centre at Fort Detrick (for a study of a fungal disease in 1967) and there were other links including 'efforts to avoid duplication'.

The committee were also told that the Stockholm International Peace Re-search Institute (SIPRI) had reported that biological warfare could be con-ducted through infected mosquitoes. (It was not told that it is only the females that pick up and transmit viruses, whereas the unit's field work has been exclusively concerned with males.) The committee concluded 'it is likely that the ultimate and only beneficiary of the GCMU (*sic*) experiments is the US military machine . . . The benefits, if any, that are likely to occur to India are not immediate but only potential.' The last sentence needs no comment. WHO has now pulled out.

We would be the first to admit that the thought of the US government using an intermediate organisation to learn how to infect Indians with yellow fever is utterly revolting. We would also admit that in military matters, particularly those of espionage, the standards of proof of involvement have, of necessity, to be lower than a judge, or a scientific referee, would deem adequate. But in this case the chain of logic is tenuous in the ex-treme: Fort Detrick has been known to collaborate with the US Public

Health Service; the USPHS represents the United States in dealing with WHO; WHO does some research in India on *A. aegypti* males; *A. aegypti* females could transmit yellow fever; yellow fever could be a biological weapon against India; therefore Fort Detrick is using the WHO to study the possibility of biological warfare in India. Perhaps the committee have more compelling evidence than they have printed. If so they should publish it at once.

That an enquiry could be conducted in this way should be a matter of con-cern to Indian scientists. For not only did the committee seem prepared to look for the worst in everything, but also if a spirit is abroad which looks with suspicion on anything with the remotest possible military connection, then India will find few opportunities to collaborate with the rest of the scientific world. Almost everything has its military aspect, and for good measure the committee suggested in-tense scrutiny headed by the Scientific Adviser to the Ministry of Defence on collaboration in

● oceanography
● meteorology
● remote sensing
● microbiology, epidemiology, ecology and virology
● toxicology
● propagation of radio waves
● science in border areas, such as Himalayan geology.

But WHO equally responsible

If one cannot but deplore the way in which WHO has had to pull out of such an important project and leave it to ICMR as best it can afford to carry on, the blame is really WHO's as much as anyone's. If it had not been for the totally leaden-footed way that the organisation has behaved, the unit would still be alive and well. It has shown itself completely incapable of handling public relations in a way to prevent a relatively small matter in-flating to gigantic dimensions. In 1973 it should have pursued a policy of being open with the Indian press—open about failures as well as successes; once-bitten-twice-shy is not a philosophy to adopt towards the press. And yet even in late 1974, when *Nature* carried a report of the fuss that had been created by Dr Jayaraman's article, we received no spirited defence of the unit from Geneva. Only now is it likely that WHO will defend the unit publicly. Did WHO believe that it shouldn't get involved in squabbles in the press? Obviously WHO has politi-cal problems in dealing on a country-by-country basis with controversy. But if it cannot be more nimble in dealing with criticism, the organisation's influ-ence is bound to diminish. ☐

international news

THIS has been a long, hot summer for the National Science Foundation (NSF), the agency chiefly responsible for supporting basic research in the United States.

For several months, NSF has been the target of mounting criticism from some members of congress, who have accused it of, among other things, wasting taxpayers' money on trivial research projects and of corrupting children's minds by supporting the development and marketing of controversial school science curricula. Such attacks are nothing new—in fact, they have been breaking out sporadically since the mid–1960s. But this time, instead of petering out, the criticism has lately turned into a broad assault on the entire process by which NSF determines which research grants to support, and which to turn down. Some issues of vital importance to university scientists are involved.

The matter finally came to a head during the past two weeks when NSF officials were called before a sub committee of the House of Representatives to listen to, and answer, a string of complaints about the manner in which NSF operates the so-called peer review system. Peer reviews, the process in which grant proposals are assessed for scientific merit by scientists outside the NSF, is used in some shape or form by virtually every government agency which supports academic research. It has never lacked critics, but the system has always been stoutly defended as the best, and probably the fairest method available for judging the relative merits of competing grant proposals.

NSF itself handles some 21,000 grant proposals each year, less than half of which are funded. Nearly three quarters of the proposals are sent out by mail to several scientists to review, and about a third of those are also reviewed by a panel of scientists at a meeting. The rest are reviewed by a panel only, frequently with the grant applicant along to explain and discuss his proposal. In 1974, each proposal on which NSF took action received an average of 6.4 reviews by scientists outside the agency.

Peer review will undoubtedly survive this latest assault, but it is already clear that some important changes will be made by NSF in the next few months. The nub of the matter is how secret the system should be. NSF's critics have been arguing for complete

NSF grants system under attack

by Colin Norman, Washington

Conlan : chipping away

openness by insisting that peer review reports and the reviewers' names should be made public, while NSF officials maintain that the system will break down unless some degree of anonymity can be guaranteed to the reviewers. The arguments have a familiar ring to the editors of learned journals.

At present, it is NSF policy that grant applicants can be given only paraphrases of the reports of scientists who review their proposals, but, at a meeting last month, the National Sciences' Board, NSF's governing council, decided that beginning next year, verbatim copies of the entire peer review reports will be made available to grant applicants on request. The board suggested, however, at least for the time being, that the names of the reviewers should be kept secret, and that the reports should not be given to a third party.

The new policy should at least

ensure that unsuccessful grant applicants will be informed of the chief reasons why their grant proposals were turned down, but the move certainly hasn't stifled the criticism. That was plainly evident when NSF officials and their critics met at the witness table during the congressional hearings last week.

The hearings began with a long, vituperative attack on NSF by John B. Conlan, a Conservative Republican from Arizona who has been chipping away at NSF for months because of its sponsorship of a few controversial school science courses. Calling the peer review process "an incestuous buddy system that frequently stifles new ideas and scientific breakthroughs, while carving up the multi-million dollar federal research and education pie in a monopoly game of grantsmanship," Conlan argued that the secrecy of the system makes it prone to abuse. It is a "completely arbitrary system that is closed and unaccountable to the scientific community and to the Congress," he said.

His line of attack was that NSF officials have too much power in deciding which projects should be funded, and that unless peer review reports are made available outside NSF, their decisions cannot properly be checked. "It is common knowledge in the scientific community that NSF programme managers can get whatever decision they want out of the peer review system to justify their decision to reject or fund a particular proposal," he said.

Citing some of his now unsuccessful attempts to obtain peer review documents relating to a science education course which he called "a new height in science porno literature," Conlan argued that the peer review process is "a sick system crying out for reform." His suggested reform is simple enough: "reviews and reviewers' names must be available to principal investigators and to the Congress. And a credible system must be devised for selecting peer reviewers that will completely divorce programme managers from the suspicion — sometimes warranted — that their own biases, prejudices, and special relationships dominate grant award decisions."

The demand for total openness in the system didn't go down all that well with members of the sub-committee, and it was strongly resisted by NSF witnesses. Apart from the traditional

argument that if reviewers cannot be guaranteed anonymity, they will either be reluctant to participate in the review process, or they will mute their criticisms, James Symington, the sub-committee chairman, suggested that such an open system would be potentially subject to political manipulation. "I believe that you would have a near 100 per cent adversary process generated by what you are suggesting," he told Conlan, and he pointed out that if the reviewers' names are made public, they could be subject to pressures, perhaps even from congressmen representing grant applicants in their districts.

The next critic of NSF to take the witness stand was Robert E. Bauman, a Conservative Republican congressman from Maryland, who earlier this year succeeded in persuading the House to pass an amendment to an NSF Budget Bill, which would enable Congress to review all NSF grants before they are awarded, and veto those it doesn't like. Though the fate of that amendment was uncertain at the end of last week—it was passed by the House but not by the Senate, and no compromise has been reached on the matter—the fact that it was approved by the House is a fair measure of the hostility toward NSF which has been building up in that august body.

Bauman last week argued that NSF's granting system is open to several criticisms. First, NSF has been supporting some trivial research projects which are a waste of taxpayers' money. Second, its work often overlaps with that of other agencies, and there could well be wasted money on duplicated studies. And, perhaps most important, it tends to stifle creative, but unorthodox views. Bauman claimed that he has received several letters which suggest that "the availability of NSF grants gives the stand-pat scientific establishment much more money and power to suppress unorthodox ideas than has ever been the case before." He suggested that more Congressional review of the agency is in order, and that "the peer review system must be opened up to the light of day."

Symington asked Bauman if he would provide the committee with copies of the letters containing criticisms of NSF. Bauman said he would be happy to do so, but he had some reservations about putting his correspondents' names in the public record. The irony was not lost on the committee.

NSF director H Guyford Stever and deputy director Richard C. Atkinson then took up NSF's side of the case They came armed with reams of computer information about how NSF's peer review system operates (see box), and argued that the process "is working with a considerable degree

One of the central complaints about NSF's Peer Review system, which has been raised in the past few months, is that the system is simply an "old boy" network which is strongly biased in favour of the large, elite universities. The suspicion, raised chiefly by Senator William Proxmire, is that the prestigious institutions not only get more than their fair share of grants, but they also provide more than their fair share of grant reviewers.

In anticipation that such complaints would inevitably surface during the hearings on Peer Review which have been taking place before a House sub-committee during the past two weeks, NSF deputy director Richard Atkinson arrived at the hearings armed with a computer analysis of NSF's granting decisions in 1974. The figures both support and refute the critics' assertions, depending on how they are interpreted.

They show, for example, that grant applicants from institutions ranked in the top 20 by the American Council on Education stand a better chance of success than their colleagues in less star-studded universities. But that is not too surprising since the institutions are considered prestigious because they supposedly have a more innovative and distinguished faculty. Atkinson noted, moreover, that "the NSF data clearly indicate that proposals submitted by scientists from the top 20 departments have the same distribution of reviewers as proposals from other schools. The assignment of reviewers in terms of the eminence of the university with which they are affiliated is not statistically correlated

with the eminence of the school from which the proposal originates." Similarly, there is no correlation between the geographic locations of reviewers and the scientist submitting the proposal.

NSF also took a look at the distribution of its funds between the states, to see whether the spread of funds is correlated with such measures as population, numbers of scientists, average income and so on. It found that three States—California, Massachusetts and New York—get more than their fair share of NSF funds, according to those criteria. Since those States contain such elite institutions as the University of California, Stanford, Harvard, MIT and Columbia, it can be argued that there is obvious bias in the system.

But, if the distribution of funds and reviewers is assessed against traditional measures of scientific excellence, such as the number of members of the National Academy of Sciences, or the numbers of former NSF graduate award holders in each State, then California and Massachusetts can argue that they are being discriminated against.

"Obviously NSF's distribution of funds turns out to be something of a compromise between a state's population and its collection of scientific talent," Atkinson noted last week. He added however that NSF has no precise formula for making this compromise—rather the various forces operating on NSF have defined this policy.

The policy will, of course, be viewed differently by different people.

of effectiveness." Stever conceded, however, that "it is true that it is possible to find specific cases where there are problems."

Stever noted that one reason why the peer review process is now coming in for considerable criticism is that "the absolute level of basic research activity supported by the federal government is inexorably declining" as the research budget is eroded by inflation. The upshot is that good grants are being turned down. "Let me ask you rhetorically," Stever said, "what is the reaction of a highly competent scientist, who intuitively knows that the proposal he submitted would produce well done, imaginative research, the results of which almost certainly would be a useful contribution to an advance in his discipline, when he subsequently is declined? True, he knows that the competition is rougher than it used to be, but he still cannot help but harbour the suspicion that somthing went wrong in the decision-making process."

NSF's new policy of making peer review reports available to grant applicants should help to remove some of that suspicion, particularly if unsuccessful applicants are given a chance to rebut criticism which they feel is either biased or invalid. But NSF will clearly find that it will be difficult to explain why grant applications with generally favourable reviews have been turned down. Atkinson noted that "NSF will have to document its decisions more carefully in order to explain to the scientific community why high quality proposals are not funded. This will be costly and will bureaucratise the decision process," he suggested, "but it may be unavoidable given the attitudes of suspicion and distrust that exists in the country today."

Be that as it may, there's clearly going to be a continuing debate about whether or not NSF should take the next step in opening up its proceedings, by making the names of peer reviewers public as well.   □

ENVIRONMENT minister Anthony Crosland cancelled the Channel Tunnel project last winter, yet an advisory group headed by Sir Alec Cairncross (Master of St. Peter's, Oxford) and originally briefed in April 1974 reported on the economic prospects of the Chunnel only last week ("The Channel Tunnel and alternative cross Channel services"). The expense of completing the assessment and publishing the report so many months after the horse had bolted would be justified if there were anything conclusive about its findings—its remit was amended to provide guidelines for assessing cost benefit if the project was revived, but Mr Crosland in the Commons on publication day turned that down emphatically.

The report, however, cannot say "indisputably" that a Chunnel would be "better than the expansion of existing services" to meet the certain growth of traffic, especially passengers and cars. This is partly through the lack of reliable data on the Tunnel-London rail-link, and British Rail is further criticised for lack of imagination both in planning with Continental partners a high-speed alternative to the air-linking of London and Paris and over devising connections with the inter-city services within Britain. In addition, it has failed fully to exploit its cross-Channel ferry services—both conventional and hovercraft, the two profit-makers on BR's otherwise red balance sheet.

Previous studies have been unbalanced through ignoring the hovercraft factor. Hovercraft now handle 30% of the passenger traffic on the short sea routes and their potential both there and on the Channel more widely is nowhere near fully developed. The British hovercraft industry should make capital out of the report since it is currently pressing for some firm orders from British Rail (Seaspeed) for a second generation of cross-Channel hovercraft to meet the needs of the 1980s onwards. Moreover if this move is turned down there may be a permanent loss of lead in hovercraft technology (and ultimately manufacture) abroad. In other words, British Rail may be operating a Channel hover-ferry service with either French or American craft in five years' time. Chauvinism has no part in the argument but good sense and timely investment have, and at the present time Britain's hovercraft industry and expertise is recognised as a European even a Western asset and brings in good export money from Iran and other OPEC areas. Big hovercraft for open sea performance is where the technological growth and profits reside, so a good deal more than an alternative to a nineteenth century engineering

fantasy hangs on the £5 million decision sought this summer.

● The Rothschild customer-contractor principle consecrated in the Government's 1972 Policy paper "A Framework for Government R & D" removed substantial sums from the direct control of the research councils to 'contractor' departments as commissioned research. Inevitably the research councils most affected—ARC, MRC and NERC are being obliged to hawk

Round Britain

their wares now to attract commissions for the work they want to do or even to complete research programmes half done. A bold example was the recent attempt by the director of the MRC Blood Pressure Unit (Glasgow) to pressurise the Department of Health through press promotion to set up a massive screening programme for people at risk from heart attacks in middle life. A two-hour MRC press briefing the day before the MRC specialist panel met to extend the current MRC funded pilot screening project for a final year described the programme and its achievements in great detail, and discussed the possibilities of a nation-wide screening programme costed at £6 million over 5–6 years. It turned out that it was not in MRC's power to take up this programme but the Department of Health could, when the pilot project was complete. If and when they do, they are likely to lean heavily on the Glasgow Blood Pressure Units' unique expertise.
● The year ending in March 1975 was one of adaptation to altered circumstances rather than of any great progress for the Medical Research Council. That is the main message of the MRC Annual Report which has just been published by HMSO. Adaptations have consisted of the post-Rothschild administrative variety, internal changes necessitated by the Council's recent reorganisation and, perhaps most importantly, changes imposed by the financial difficulties of the universities.

Total expenditure by the MRC last year, including £5.5 million coming by way of the Health Departments and the Department of Employment, amounted to £36 million This represented a reduction of 1.7% in real terms compared with the previous year. An oddity of the financial outgoings of the MRC was that less was spent

on grants last year than expected. That was due partly to unusually late applications and partly to a growth in unfilled positions for research assistants in universities. The dual-support system of funding university research has been under great pressure with the universities increasingly unable to provide their share, namely the salary of the principal investigators, laboratory space and ordinary departmental overheads. Sir John Gray, secretary of the MRC, stated his recognition that the situation was bound to deteriorate further but added that he felt the MRC had sufficient flexibility to cope, partly by implementing a modest capital development programme for university research.

The MRC Research Groups are one casualty of the present stringencies. In the past they were set up when a university wished to pursue a particular line of research and agreed to assume financial responsibility for the Group after a limited initial period of MRC support. Universities can no longer afford to give undertakings like that and no new Research Groups were established last year.

The past year has also seen a new career structure for the scientific staff of the MRC With less numerous and flexible careers available in universities, the MRC has been persuaded to offer far greater opportunities of tenure within its own establishments, whilst regretting the consequent reduction in the mobility of its scientists. The Cell Biology and Disorders Board of the MRC has set up two sub-committees to review specific fields of research. One, under Professor W. F. Bodmer, will be looking at the field of clinical genetics including the possibilities for screening programmes. The other, under Professor D. C. Phillips, will review molecular biology with particular emphasis on the role of the Laboratory of Molecular Biology in Cambridge. And the Council is continuing to evaluate last year's Neuberger report on Food and Nutrition Research.

● THE Committee of Vice-Chancellors and Principals introduced the first report of a study group on postgraduate education—more in the nature of a Green Paper than a White one, floating ideas for discussion in the universities in place of a hardcore of recommendations intended as policy. Nothing highly controversial in the group's findings, except perhaps a suggestion that overseas students should be asked to pay more for education at British universities than native scholars. When the government first introduced differential fees in 1966, most universities were reluctant to make the charges, and some flatly refused, but there have been changes in the status

INDIA has forced her way out of the exclusive smallpox club. From June 30, there was not a single indigenous case of smallpox in the country, according to figures received from the field from all sources including the World Health Organisation (WHO). This has been hailed as a remarkable feat. Only last year, nearly 200,000 cases with more than 30,000 deaths were reported from all over the country. Six states—Bihar, Uttar Pradesh, West Bengal, Assam, Orissa and Madhya Pradesh—accounted for nearly 99% of the cases, with Bihar leading them all with a staggering 68% of the total.

Starting in October 1973, concerted and intensive efforts were undertaken jointly by the government of India, the state governments, and the WHO to tackle the disease. Health workers literally visited each and every village in the endemic areas to inquire about smallpox cases, and as a result of hard and dedicated work, in less than two years, the disease has been wiped out.

Congratulating the workers and agencies whose efforts made this achievement possible the Union Health Minister, Dr Karan Singh, also announced that the vaccination programme would be continued for another two years to ensure against recurrence of the disease. Unfortunately, smallpox has not yet been controlled completely in neighbouring Bangladesh and there is always the danger of its importation from across the border. The government, Dr Karan Singh emphasised, was well aware of this and had already stepped up vigilance arrangements on the border with Bangladesh, so that no smallpox cases entered the country undetected. India is self-sufficient in the production of the preventative smallpox vaccine and has already donated 275,000 ampoules to Bangladesh; more will be made available to that country, if necessary.

Meanwhile, the Health Minister also announced that the award for reporting of any fresh incidence of smallpox from July 1, 1975 would be Rs.1,000—ten times what it used to be.

● India's first satellite, Aryabhata, has been orbiting the Earth for over three months now. The data being received indicate that all systems and instru-

Indian diary

from Narendar K. Sehgal

ments of the satellite are healthy and functioning normally. The three scientific experiments on board had to be switched off, however, after the first five days, following detection of a fault in one of the four lines delivering power to the experimental packages.

Scientists at the Indian Scientific Satellite Project at Peenya (near Bangalore) say they have good data from two of the three experiments— the one looking for X rays in space and the other aimed at detecting high-energy neutrons and gamma rays at times of intense solar activity— received during the first five days, but none from the aeronomy experiment which was to look for electrons in the ionosphere and ultraviolet radiation in the night sky.

As soon as the fault was detected, all scientific experiments were turned off for fear that the trouble might spread to the remaining three power lines as well. Using simulation techniques, the scientists have been working overtime to determine and pinpoint exactly what went wrong. So far, they have not succeeded. Redundant instrument capacity was not built into the experimental packages for several reasons. The principal one was that the experiments were only a secondary objective of the Aryabhata mission. The primary aim was to establish satellite fabrication capability and to see if the various systems and components aboard functioned and performed as planned.

Indian space scientists have not given up all hope yet; they are planning to turn on the experiments again, hoping that the fault—possibly due to some stuck relay which prevents power flow in one of the lines—may have corrected itself. They do admit, however, that chances of this happening are rather slim. In the meantime, studies will continue unabated to determine the exact cause of the failure.

As if to counter this hitch, there has been an unexpectedly happy development too. The operational life of Aryabhata was originally estimated to be about six months. That was how long the inert gas supply on board (used in the satellite's stablisation mechanism) was expected to last. But fresh calculations, based on actual data being received, show that the gas supply would last much longer, possibly more than 18 months. This will give scientists extra time in which to determine and, if possible, correct the fault and revive the experiments.

Encouraged · by the success of the first attempt, the Indian Space Research Organisation has already signed an agreement with the Soviet Union to launch India's second satellite, Aryabhata-2, in 1977–78. The second satellite is expected to be a lot more sophisticated and it will carry television camera systems to survey mineral deposits and agricultural crops, among other things. The flight model for Aryabhata-2 will actually be a modified version of the back-up model of the one now in orbit.

and capabilities of countries sending students to Britain in the interim which are likely to ensure a milder response to the Vice-Chancellors' proposal.

Ten years ago the majority of overseas students came from relatively poor countries which were in no position to provide postgraduate education themselves and the facilities provided in Britain were seen as a relatively inexpensive form of aid. But these days, says the study group, many students come from countries which in terms of national income per head of population are better off than we are, and it is clearly arguable that in these cases the fee payable should be at least a significant proportion of the cost of providing the course.

The fee would not have to be so high that it was out of proportion with similar charges in Western Europe or the US (which would leave plenty of leeway with MIT charges running at $4,000 to $5,000 a year), and the needs of the countries which are still underdeveloped could be met by a system of postgraduate scholarships, continuing the notion of offering development aid in the form of education. Even so, the first effect of any increase in charges would almost certainly be a fall in the number of overseas students arriving in Britain.

The universities want to keep the door open to foreign students not only because of the idea of a world academic community, visiting and returning visits internationally, but also because there is a theory that scientists and technologists trained in Britain will one day be ordering British goods when they're sitting at the biggest desk in the headquarters of the Zambian State Uranium Corporation, or Burundi Rubber. An unfortunate flaw in the theory is that British industry doesn't seem to recognise the value of postgraduate education, with the result that in certain fields it doesn't achieve the sophistication which can be managed by countries (like Germany) which automatically look for masters degrees and doctorates in recruits to their engineering industries. A member of the Vice-Chancellors' group confessed, privately, that at CERN, for example, British loyalists were wringing their

hands and looking elsewhere for equipment, simply because we don't have the calibre of engineer to produce the sort of equipment they require.

Not surprisingly then, the study group is recommending that steps should be taken to see that British industry becomes acquainted with the meaning and value of the masters degree. Also they suggest a tightening up of the admission procedures to a PhD course, in view of justified criticism to the effect that less than ideal candidates are being accepted, and in some cases are receiving the degree. To weed out the stragglers before they have gone too far, the Vice-Chancellors will be asking universities to consider the introduction of a more formal and rigorous assessment of progress, possibly in the form of a written examination or preliminary dissertation, at the end of the first year of a PhD course. And by way of consolation for

the candidates who get over this additional hurdle in the education race, there is a recommendation that a special mark of distinction might be considered for outstanding theses. So if all goes well it will only be a matter of time before the plushest jobs are reserved for thinkers with an upper second PhD or better.

Perhaps by way of a reply to Lord Crowther-Hunt, the report steered clear of making recommendations about the relation between student places and manpower requirements. Apart from areas like medicine and law, where there is a clear connection between the number of career openings and university places, the Vice-Chancellors suggest that in general, progress to postgraduate education should be on the principle that any student who is "qualified, suitable and keen to proceed" beyond first degree level should be able to do so.   □

This week we're running the first in a series of competitions designed to allow scientific minds a flight of literary fancy. Two or three weeks' leeway will be allowed for overseas entrants, but UK competitors are asked to submit their efforts within a week of publication. £10 is available for the best entry or entries

No. 1: Dissident scientists in the Soviet Union are forced to confine their activities to the meetings of the famous Sunday seminars A prize for the minutes (not more than 150 words) of an illicit seminar somewhere in the Western world.   □

correspondence

Sulphones for leprosy

Sir,—If Drs. Browne and Davey (*Nature*, May 15) agree with my statement (March 20) that "sulphones provide a cheap and practical form of treatment", why are most of the World's leprosy patients not receiving it? In countries or areas adopting the outpatient approach, the percentage on treatment is very high; for example, in an area of Northern Nigeria where I worked about 90%; in a remote area of New Guinea 100% (*Med J. Anat.*, **1**, 1262, 1971) and in an area of India, 60% after only three years of starting outpatient clinics (*Dan. Med. Bull.*, **20**, 198, 1973). In countries persisting with institutional care, such as Tanzania, only 33% receive treatment, and some patients have to travel up to 250 miles to receive it. Rehabilitation, including reconstructive surgery, prostheses, physiotherapy and occupational therapy, has greatly magnified the cost of institutional care. Vast sums have been spent on a rehabilitation centre (ALERT) in Ethiopia for crippled patients, yet only 40% of patients are receiving dapsone. Thus, it is the strategy employed which determines the number on treatment and not economic reasons or social aspects of the disease. Will Mr Van Den Wijngaert (May 22) tell us how much of the £4 million collected by the European anti-leprosy Association (ILEP) in 1973 was spent on institutional care, including rehabilitation, and how much

on outpatient treatment?

The figures I quoted showing the rapid fall in leprosy incidence in areas where sulphones have been used, have been ignored by Drs. Browne and Davey. LEPRA have not published any incidence figures in their Malawi project, which started nine years ago, and ILEP have not used money for this purpose. Thus, it is a failure of evaluation, rather than a failure of sulphones to rapidly interrupt transmission, which is the problem. A proved and practicable method of prevention is available and no more time should be wasted on BCG or chemoprophylaxis. Immunoprophylaxis using dead M. leprae from infected armadillos, as suggested by Drs Browne and Davey, has no proven preventive effect.

If government are informed of these facts, if voluntary agencies spend the money on treating patients before they become crippled and if WHO arranges for proper evaluation, then all leprosy patients can be treated and the disease could probably be eradicated within the next ten years.

C. L. CRAWFORD
*Department of Anatomy and
  Embryology*
University College London.

North to Alaska

Sir,—In her correspondence "North to Alaska" (May 29) Angela Croome states that no ship got through the North

West passage until 1944. This is not quite correct. During the years 1903–06 the Norwegian explorer Roald Amundsen succeeded in getting his little ship Gjøa through. Thus he is rightly credited with the "discovery" of the North West passage.

Yours faithfully,
                    K. STENSTADVOLD
University of Trondheim, Norway

A hundred years ago

THE recent French inundations have recalled to memory an experiment which was tried twelve years ago before Napoleon III. The design was to manufacture mattresses of cork, so that any one on board a ship or in a house which could be flooded would have in his bed a ready-made raft capable of floating under a weight of more than 1 cwt. for any length of time. Cork is a material so soft that mattresses made of it are not inferior to any other for comfort.

from *Nature*, **12**, 280; August 5, 1875.

news and views

Rhizobium as a free-living nitrogen fixer

from John Postgate

SCIENTISTS interested in biological nitrogen fixation have for many years been perplexed by the apparently complete dependence of Rhizobium, the bacterial partner in the well-known legume symbiosis, on its host plant. Despite frequent attempts nobody, until about six months ago, had successfully obtained growth and fixation by rhizobia in the absence of living plant material (either as a nodulated plant or an association between rhizobium and a tissue culture of plant callus). The plant, indeed, contributes genetic information important to the nodule symbiosis, so Dilworth and Parker (J. theoret. Biol., 25, 208; 1969) earlier suggested that some of the genes determining rhizobium's ability to fix nitrogen (nif genes in geneticists' shorthand) were actually 'banked' with the host plant. More recently evidence had been accumulating to support the contrary view: that rhizobia possess the complete quota of nif genes themselves but that the genes are silent in free-living cells.

One line of evidence stemmed from the work of Dunican and Tierney (Biochem. biophys. Res. Commun., 57, 62; 1974) who took a strain of Klebsiella aerogenes which did not normally fix nitrogen, transferred genetic material from Rhizobium trifolii to it, and obtained progeny which fixed. The presumption was that the K. aerogenes Nif$^+$ progeny were using rhizobial nif genes but, as the authors recognised, ability to fix nitrogen is common among klebsiellae and the possibility that K. aerogenes possessed cryptic (latent) nif genes, and that some activator had been transferred, was not excluded Circumstantial evidence came also from H. J. Evans's group, who showed that R. japonicum grown in conventional laboratory culture, in the absence of plant material, possess not only the electron transport factor associated with nitrogenase (Plant Physiol., 51, 136; 1973) but also a protein immunologically similar to the molybdenum-iron protein of nitrogenase (Biochim. biophys. Acta, 381, 248; 1975). Even stronger evidence was provided by the experiments published in Nature a few months ago (see Nature, 253, 305; 1975) in which

strains of the slow-growing cowpea type of rhizobium were shown not only to fix nitrogen in the presence of callus from non-leguminous plants, but also to continue fixing nitrogen for up to twelve hours after the callus was removed. Callus was clearly providing a diffusible material which permitted nitrogen fixation by the rhizobia; it might be a genetic activator, but actual gene transfer was hardly likely, particularly since non-leguminous callus functioned as well as legume callus in the artificial symbioses.

In the event, the situation has proved remarkably simple. In this issue of Nature three groups, two from Australia and one from Canada (pages 406, 407 and 409) simultaneously report successful cultivation of nitrogen-fixing rhizobia in the complete absence of plant material. As with the earlier experiments using callus culture associations, the possibility that the rhizobial cultures might be contaminated by free-living nitrogen-fixing bacteria had to be rigidly excluded, because rhizobia are difficult to sustain as pure cultures, and the slow growth of the cowpea group makes them even more awkward in this respect. In addition, positive acetylene tests needed to be backed up with $^{15}N_2$.

These things have been done and the key to the question proves to be the carbon source: for fixation, a pentose such as arabinose or xylose as well as a dicarboxylic acid such as succinate seem to be essential Note that both classes of carbon source are common plant constituents. A relatively small amount of fixed nitrogen (such as glutamine, glutamate or nitrate) seems to be helpful; in this respect the cowpea rhizobia seem to resemble free-living aerobic nitrogen-fixing bacteria such as Derxia gummosa or Mycobacterium flavum which also fix nitrogen best when 'kicked off' with a little pre-fixed nitrogen. In D. gummosa, the need for fixed nitrogen can be simply interpreted: nitrogen fixation is an oxygen-sensitive process, so a little pre-fixed nitrogen permits colonies or cultures to grow to a density such that, within the colony, the cooperative respiration of the bacteria lowers the oxygen tension to a level at which fixa-

tion can occur (Hill, J. gen. Microbiol, 67, 77; 1971).

So far, all the successful experiments with rhizobia have made use of media set with agar, on the surface of which the colonies of rhizobia grow and within which colonies the oxygen tension may range from zero to atmospheric. Since fixation by rhizobia has long been known to involve aerobic metabolism, it is tempting to assume that the cowpea rhizobia, like D. gummosa and M. flavum, are bacteria which become microaerophilic when fixing nitrogen. There is an apparent paradox, brought out by the Canadian group, that nitrate or ammonia, which repress nitrogen fixation in liquid cultures of ordinary fixers such as clostridia, azotobacters or klebsiellae, actually promote fixation by free-living rhizobia, but the local ammonia or nitrate concentration near an agar colony cannot readily be assessed and it would be premature to conclude that regulation of nif in these rhizobia is unusual. Resolution of both of these problems must await the successful culture of nitrogen-fixing rhizobia in homogenous liquid media, where both oxygen and fixed nitrogen concentrations can be measured. No doubt this is only a matter of time.

The substantial advance represented by this work is not only the final proof that cowpea and some other rhizobia carry the complete complement of nif genes; it is also the fact that many strains and species of rhizobia now join the ranks of free-living nitrogen-fixing bacteria, with revolutionary consequences for the study of their biochemistry and genetics—for one thing, the host plant can be by-passed in the laboratory. A well established obligate symbiosis is crumbling; in what does the host specificity of the traditional legume symbioses reside? And is the nodule nothing more than a compartment to restrict access of oxygen to rhizobia? If only a pentose and a dicarboxyclic acid are needed for rhizobial fixation, how readily can this information be used to set up new associations with plants and forage crops? And how many other free-living nitrogen-fixing bacteria have been missed by microbiologists because two carbon sources are needed?

Degrees of antiquity

from Peter D. Moore

PRIMITIVE things have a fascination all of their own; and when those primitive things, are, in addition, both rare and attractive, it is natural that they should become a source of attention. All of these things are true of the cycads, which, being the sole survivors of a group that was widespread and successful during the Mesozoic era, were romantically termed 'living fossils' by Chamberlain in 1935. Although they are not considered to be in the direct line of Angiosperm evolution, they probably represent an offshoot from it and it is natural that modern taxonomic techniques should be brought to bear upon the problems of their internal and external relationships.

There are three extant families of cycads, the Cycadaceae, Stangeriaceae and Zamiaceae, and views have differed regarding their relative primitiveness. Sporne (*Morphology of Gymnosperms*, Hutchinson, London; 1965) considered *Stangeria* to be most primitive in that it possesses the largest number of fernlike characters, whereas Marchant (*Chromosoma*, 24, 100; 1968) preferred to regard *Cycas* and *Microcycas* as primitive, both on morphological and cytological grounds. In his cytotaxonomic work on cycads, Marchant examined chromosome number and form in 35 species belonging to eight of the ten extant cycad genera. He found that many species possess telocentric chromosomes (that is, having a terminal centromere), a character which he regarded as primitive. High chromosome number he also considered a primitive feature. On this basis *Microcycas* (Zamiaceae; 2n=26,. 22 telocentric) and *Cycas* (Cycadaceae; 2n= 22, 20 telocentric) emerge as particularly primitive. *Stangeria* (Stangeriaceae) has a chromosome complement in which 2n=16, two only of which are telocentric. Marchant, however, admitted that it was impossible to refute the reverse argument, that telocentric chromosomes represented a derived, or advanced condition. The extreme antiquity of the genome, of course, makes interpretation difficult since there has been ample time for complex evolutionary processes.

Dossaji, Mabry and Bell (*Biochem. System Ecol.*, 2, 171, 1975) have now made a chemotaxonomic approach to the problem of evolutionary relationships among the cycads by analysing biflavonoids from their leaves. Fourteen of these compounds, which are restricted mainly to gymnosperms, were identified in the 82 species studied. Ten species of *Cycas* were uniform in their biflavonoid patterns and contained some biflavonoids, such as hinokifla-

vone, which are not found in the other two families. Within the Zamiaceae, the pattern is slightly more diverse, and both of these families have biflavonoids in common with *Ginkgo biloba*, another surviving member of a primitive group of gymnosperms. Perhaps the most striking result, however, is the complete absence of biflavonoids from the leaves of *Stangeria*. Dossaji *et al.* regard this as a derived condition in which the capacity for biflavonoid synthesis has been lost as has happened in the Pinaceae. Thus, in spite of its primitive morphology, *Stangeria* is beginning to achieve the status of an advanced cycad.

It would have pleased Chamberlain to know that it was plants collected by himself and now cherished by Professor Bold in Texas which provided the incentive for these studies.

Mapping viral integration sites with somatic cell hybrids

from J. K. McDougall

CELLS transformed by viruses into a potential or actual malignant state can usually be shown by molecular hybridisation techniques to retain at least part of the viral DNA molecule covalently linked to cellular DNA. One of the phenotypic changes which these altered cells exhibit is the expression of antigens which are virus specific, for example the tumour antigens (T-ag) and transplantation antigens (TSTA) found in cells transformed by Simian Virus 40 (SV40) and adenoviruses. The progress made in the identification of sequences of viral DNA which persist in single or multiple copies has been spectacular since the restriction endonucleases became available Having reached a point where the viral DNA integrated into a particular cell line can be accurately described, the tumour virologist is that much closer to analysing the role of viral genes in inducing and maintaining transformation. The location(s) at which viral sequences are integrated into host chromosomes may be of prime importance in this analysis.

Croce and his co-workers (Croce, Girardi and Koprowski, *Proc. natn Acad. Sci. U.S.A.*, 70, 3617; 1973) made an encouraging start to answering the question of whether specific integration sites exist, by making use of gene mapping techniques in somatic cell hybrids (Weiss and Green, *Proc. natn Acad. Sci. U.S A.*, 58, 1104; 1967). Hybridisation of SV40-transformed human cells with thymidine kinase negative mouse cells resulted in selection of hybrid cells in which reten-

tion of human chromosome C7 and expression of SV40 T-antigen, TSTA and rescue of defective virus were concordant (Croce, Huebner, Girardi and Koprowski, *Virology*, 60, 276, 1974). Fusion of mouse peritoneal macrophages, which do not divide in these *in vitro* conditions, with the virus-transformed human cells resulted in cell lines which were all transformed and which all contained C7 (Croce and Koprowski, *J. exp. Med.*, 140, 1221; 1974).

Thus the criteria for gene assignments to chromosomes (Ruddle, *Nature*, 242, 165; 1973) appear to be satisfied, allowing Croce and Koprowski (*Proc natn. Acad. Sci. U.S.A.*, 72, 1658; 1975) to conclude that the gene(s) responsible for transformation and the integrated SV40 genome are located on C7.

The study has been taken a step further (Croce, Aden and Koprowski, *Proc. natn. Acad. Sci. U.S.A.*, 72, 1397; 1975) by injecting the transformed hybrid cells into nude mice, which act as convenient culture hosts for heterotransplants (Rygaard and Povlsen, *Acta Pathol. Microbiol. Scand*, 77, 758; 1969) Inoculation of an uncloned population of hybrid cells, which all contained chromosome C7 together with different frequencies of 13 other human chromosomes, resulted in the development of tumours. Analysis of the tumour cells showed 100% retention of C7 and expression of SV40 T-antigen, but a reduction, often complete, in the frequency of all other human chromosomes. Although most cloned hybrid cells and the tumours derived from them were near tetraploid for the mouse and contained on average three C7 chromosomes, more recent studies (Croce, Aden and Koprowski, *Science*, in the press) have shown that hybrids with a near diploid complement of mouse chromosomes and only one C7 are tumorigenic and have the SV40-transformed phenotype.

The hybrids derived by Croce do not follow any pattern which would be compatible with the hypothesis of chromosomal balance and suppression of malignancy (see Jones, *Nature*, 252, 525; 1974) Neither the transformed phenotype *in vitro* nor tumour induction is associated with the loss of specific mouse chromosomes in the man–mouse hybrids or of human chromosomes in hybrids between normal human and SV40-transformed human cells (Croce, Huebner, Girardi and Koprowski, *Cold Spring Harb. Symp. quant Biol.*, 39, 335; 1975) In these experiments it would seem therefore that the transformed phenotype and malignancy are under positive control.

The results from Croce's studies all show that SV40 DNA integrates into

only one chromosome, in the two bio-chemically abnormal human cell lines examined. No SV40 DNA sequences could be detected using the sensitive DNA–DNA reassociation kinetics method, in any hybrid lacking C7. Results from molecular hybridisation studies on SV40-transformed mouse cells (Botchan, Ozanne, Sudgen, Sharp and Sambrook, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4183; 1974) show that the pattern of viral DNA sequences integrated in transformed cells can be very complex, in that different regions of the virus genome may be present at different frequencies. The situation with adenovirus-transformed cells is similar (Gallimore, Sharp and Sambrook, *J. molec. Biol.*, **89**, 49; 1974). Kelly and Sambrook (*Cold Spring Harb. Symp. quant. Biol.*, **39**, 345; 1975) suggest an integration of SV40 DNA sequences in more than one chromosome although expression, in terms of T-antigen and cytochalasin B susceptibility, is associated with specific chromosomes. Many SV40-transformed cell lines have been shown to contain multiple copies of the viral genome (Ozanne, Sharp and Sambrook, *J. Virol.*, **12**, 90; 1973). This is again true of adenovirus-transformed cells, although the whole adenovirus genome is not found.

Before the generalisation that SV40 integrates only into chromosome C7 in human cells can be made, it will be necessary to examine other SV40-transformed human cell lines and in particular, those containing multiple copies. Weiss (*Proc. natn. Acad. Sci. U.S.A.*, **66**, 79; 1970) has previously suggested a random association of SV40 T-antigen expression with human chromosomes. A crucial question is the role of chromosome C7 in the somatic cell hybrids and whether there may not be some growth advantage conferred by host genes located on this chromosome. These might nevertheless be under the control of viral genes. Croce has been unable to obtain hybrid lines by fusion of mouse macrophages with untransformed human cells.

Somatic cell hybridisation techniques, which have accelerated the mapping of genetic loci in mammalian genomes, must now be a method of choice for localising integrated viral sequences. The results from current studies on adenovirus-transformed human and rodent cell lines, in which the viral sequences have been quantitated, will be of great interest. Croce and his colleagues have demonstrated the power of this approach, the implications of which extend beyond the experimental situation. The recognition of transforming genes, whether of viral origin or not, should be possible using these methods.

Structure and functions of an early T7 promoter

from Maria Szekely

THE nature of promoter sites, defined originally as regions of DNA required for initiation of transcription, has attracted the interest of many laboratories Attempts to isolate these sites and to determine their structure were made as early as 1972, when Heyden, Nüsslein and Schaller (*Nature new Biol.*, **96**, 9) isolated a fragment from fd DNA, the binding site of RNA polymerase. Polymerase binding sites have since been isolated from a number of different DNAs, making use of the fact that the binding of this enzyme protects the corresponding DNA fragment from nuclease digestion. The length of the fragments obtained was in most cases around 40 base pairs, although Giacomoni *et al* (*Proc. natn Acad. Sci. U S.A.*, **71**, 3091; 1974) found binding sites 14 base pairs long on T5, T7 and λ DNA by using somewhat different conditions for nuclease digestion. Recent improved techniques for DNA sequencing made it possible to establish the nucleotide sequence of polymerase binding sites in fd (Schaller *et al*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 737; 1975), SV40 (Dhar *et al.*, *Nucleic Acids Res.*, **1**, 595; 1974), lambda P$_L$ (Maniatis *et al*, *Nature*, **250**, 394; 1974), tyr-tRNA (Sekiya and Khorana, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 2978; 1974) and lac DNA (Dickson *et al*, *Science*, **187**, 27; 1975).

The function of the promoter is, however, more complex than the simple binding of RNA polymerase. The functional definition of a promoter site is in fact somewhat vague, as it is not clear whether the same DNA region is responsible for the different stages in the initiation of transcription. The functions of the promoter include providing a recognition site for the polymerase, and providing an entry site where a stable 'closed complex' can be formed (Chamberlin, *A. rev. Biochem*, **43**, 721; 1974) which is converted into an 'open complex' in which a short stretch of the DNA strands has been separated. Only the open complex is able to start RNA synthesis. The starting point of the actual transcription process is also part of the promoter. There are, however, contradictory data and hypotheses in the literature as to whether the binding and starting sites are overlapping, or whether they are far apart, thus requiring a migration of the enzyme along the DNA. The finding of Schaller *et al* (see above) that the sequence of a strong polymerase binding site on the replicative form of fd DNA includes the 5'-

terminal sequence of the corresponding mRNA (determined by Heyden) suggested that the binding and starting sites probably overlap. Dickson *et al.* established the sequence of the whole control region of the lac operon (*Science*, **187**, 27; 1975) and determined the site of interaction with RNA polymerase by locating the nucleotide changes produced by different promoter mutants. They came to the conclusion that this interaction site does not coincide with the starting point for transcription. They propose a scheme for initiation of lac transcription according to which the enzyme moves 35 base pairs from the entry site to the start site.

The disagreement may probably be ascribed to the fact that very different approaches and some indirect methods have been applied in both the above studies. It is therefore of great interest that a direct comparison of binding and initiation sites produced more conclusive data.

A recent article by Pribnow (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 784; 1975) elucidates these problems by identifying the binding sites of RNA polymerase at an early promoter site of T7 DNA under conditions of initiated or non-initiated complex formation and by comparing the nucleotide sequence of this site with the 5'-terminal sequence of the mRNA transcribed from this promoter. Pribnow finds that the polymerase binding site is the same whether transcription is initiated or not, and that the starting point of transcription is around the centre of the binding site.

He uses specific dinucleotides to direct initiation of transcription to one or other of three early promoter sites. Specific initiation at promoter site A3 can be achieved in the presence of CpA and a very low concentration of CTP (Minkley and Pribnow, *J molec. Biol.*, **77**, 255; 1973). Under these conditions only the first three nucleotides of the mRNA are joined together, CpApC-OH is formed, and the polymerase is 'locked' into this site. The binding site of the enzyme—the fragment of DNA protected by the polymerase—can be isolated and sequenced. If, after the formation of this complex, transcription is allowed to proceed for 10 min, RNA fragments up to 80 nucleotides long can be obtained, the first 19 nucleotides of which have been sequenced. This sequence corresponds exactly to the 3'-half of the 43 base pair long DNA fragment which binds the polymerase. The sequencing of the DNA fragment itself was done by an indirect method: a stretch of RNA complementary to the DNA fragment was synthesised by read-through transcription and isolated by hybridisation techniques and its nucleotide sequence

was determined. Essentially the same technique was used to establish the structure of the non-initiated polymerase binding site. Tight, non-initiated binary complexes of DNA and RNA polymerase were obtained in low salt media in the absence of dinucleotides or nucleoside triphosphates. The protected DNA fragment was isolated and its sequence was found to be identical to that of the initiated binding site.

It is thus clear that the binding site of the polymerase does not change when transcription is initiated and that this binding site overlaps with the starting site of transcription. Further data show that the same DNA region does not fulfil the function of a polymerase recognition site. The protected DNA fragment is unable to rebind the enzyme (in agreement with the findings of Schaller *et al.*, see above), probably because some further information, not contained in the 43 base pairs, is required for recognition of the promoter site. Still, even in this known sequence, striking homologies are found with polymerase binding sequences in other DNAs, suggesting either that this DNA stretch may also play a part in the recognition process or that the stable binding of polymerase requires the presence of a short specific sequence in all promoter sites.

Very similar conclusions have recently been reached by Heyden, Nüsslein and Schaller (*Eur. J. Biochem.*, **55**, 147; 1975).

Protein conformation and α-complementation

from Roger Pain

RESTORATION of activity to an inactive mutant enzyme is always intriguing. When a purified system is developed such that the phenomenon can be translated from the realm of genetics into solid protein chemistry then one can expect interesting advances, both to the cell biologist and the protein conformation–activity industry.

Attention was first drawn to a particularly interesting example of mutant reactivation by Ullman, Jacob and Monod (*J. molec. Biol.*, **24**, 339–343; 1967) involving β-galactosidase, the enzyme specified by the *z* gene of the lactose operon in *E. coli*. Inactive mutants of this enzyme bearing mutations in a particular region of the *z* gene can have their activity restored by non-covalent binding of the gene product from *z* genes bearing mutations outside this same region. On the map of the *z* gene (see figure) both the operator-proximal α region and the
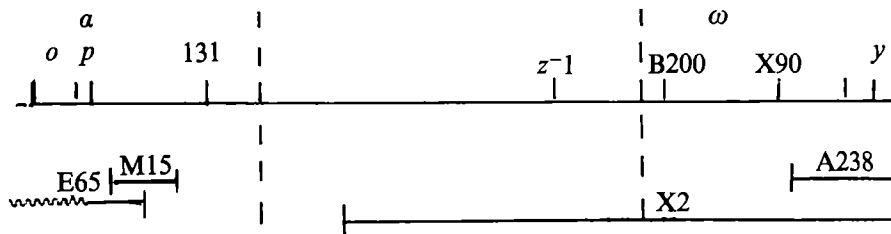
The *z* gene for β-galactosidase: figures and letters above the solid line indicate the position of point mutations in the *z* gene; *o*, operator; *p*, promoter; *y*, structural gene of β-galactosidase permease. From Ullman, Jacob, and Monod, *J. molec. Biol.*, **24**, 339; 1967.

operator-distal region specifying the N-terminal and C-terminal regions respectively of the β-galactosidase subunit can act as 'acceptors', if mutated, or as 'donors', if intact, in the reactivation or complementation 'process. As an example of α-complementation, the protein specified by the deletion mutant M15 can be restored to wild-type β-galactosidase activity by binding proteins arising from point mutations such as z^{-1}, B200 or X90 or deletions such as A238 or X2, but not by mutants such as 131 or E65. M15 is termed an α-acceptor while z^-1, A238, and so on exhibit α-donor activity. Finally, wild-type β-galactosidase consists of four identical subunits, each of molecular weight 135,000. α-complementation involves the binding of a polypeptide donor to the individual subunit, enabling it to form an active enzyme, rather than the association of different subunits; it is intra- rather than inter-cistronic in character.

A significant step forward in this fascinating phenomenon has been taken by Zabin and his colleagues who have purified the M15 protein, shown how it differs in sequence from the wild-type enzyme and also purified a sequenced peptide with α-donor activity (Langley *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 1254–1257; 1975). Cyanogen bromide cleavage of β-galactosidase yields peptides with α-donor activity; peptide CB2, residues 3–92, has been purified and found to complement purified M15 protein, giving a specific activity close to that of pure β-galactosidase. This confirms the genetic analysis that α-complementation involves the N-terminal region of the enzyme. The single CNBr peptide peculiar to M15 protein as compared with wild type β-galactosidase was found to correspond to CB2 and has been purified by an ingenious double labelling procedure and shown to differ from CB2 in that residues 11–41 are missing. It is therefore these 31 deleted residues in M15 protein whose function is supplied by the α-donor peptide CB2 in α-complementation.

An · interesting model for such systems already exists in the work of Anfinsen on staphylococcal nuclease (*Science*, **181**, 223–230; 1973). In this

single subunit enzyme of 149 residues, the tryptic fragment (1 to 126)—a protein chemist's deletion mutant—can be complemented by peptide 99 to 149 with partial restoration of enzyme activity. Further, the overlapping regions may be 'trimmed' by proteolysis in the same way that certain complemented β-galactosidase mutants have been trimmed (Ullman and Perrin in *The Lactose Operon*, edit. by Zipser and Beckwith; Cold Spring Harbor, 1970). Anfinsen's well characterised model would act as a precedent for suggesting that the peptide CB2 provides residues 1–41 in the wild-type conformation with the remainder 42–92 and residues 1–10 of M15 going spare, yet without interfering with the structural features essential for activity.

The fact that M15 protein possesses a strong substrate binding site, together with the observation of Langley *et al.* that it behaves in solution as a dimer rather than a tetramer, suggests that α-complementation may be concerned with inter-subunit binding sites. The sharp delineation of the α-region shown by genetic analysis may well reflect the boundary of a protein domain such as are now known to exist in several larger enzymes. Immunological evidence in favour of this hypothesis has recently been published by Celada, Ullman and Monod (*Biochemistry*, **13**, 5543–5547; 1974).

With the ability now to study the phenomenon of complementation on purified systems it is perhaps worthwhile speculating on one or two implications for protein conformation of the original results of Ullman *et al.* Treatment with guanidinium chloride (GuCl) was found to destroy α-acceptor activity in all mutants examined, while α-donor activity was unaffected. This suggests that the N-terminal α region must be free from deletions for refolding to occur from GuCl but not presumably *in vivo*. Further, mutants having α-donor activity can be divided into two classes: mutants in the operator-distal ω region whose α-donor activity is only revealed after exposure to 6 M GuCl and subsequent return to aqueous solution and mutants in the central region between the α and ω regions whose α-donor activity is evi-

dent both before and after GuCl treatment. Consideration of the unfolding and refolding processes involved suggests that even a minimally altered ω region may affect the *in vitro* folding of other regions or domains of the enzyme in a way different from the *in vivo* situation. It must be acknowledged that these experiments were carried out on crude extracts of enzymes and mutants. If, however, they were reproduced using purified systems like those of Langley *et al.*, they would pose most interesting and potentially productive questions about the kinetic pathways along which large polypeptide chains fold in the test tube on the one hand and off the ribosome on the other.

Mössbauer effect and order–disorder transitions in alloys

from G. Longworth

To study the mechanisms involved in order–disorder reactions in alloys, the functional dependence of some physical quantity (for example resistance, magnetisation or X-ray diffraction pattern) must be measured. An ordered alloy is one whose structure may be divided into sublattices each of which is occupied predominantly by one type of atom, whereas after disordering the various atoms are arranged at random on each sublattice. Such reactions are divided into those of first or second order (degree) involving either a discontinuous or continuous change in long-range order parameter with temperature. At a first order transition, usually associated with closely packed lattices such as in Cu_3Au or Ni_3Fe, two phases of different composition may coexist, whose transformation is governed by a latent heat. The highly monochromatic gamma rays emitted by certain (Mössbauer) nuclides may be used to study order–disorder reactions by measuring the hyperfine interactions, which are a function of local atomic order.

Thus the complex ordering in Fe_3Al has been studied by Cser *et al.* (*Phys. Stat. Sol.*, **20**, 581; 1967) by determining the changes in magnetic hyperfine field (H_{hf}) at the ^{57}Fe sites. In iron alloys, H_{hf} is determined essentially by the magnetic moment on the iron atom and by the conduction electron polarisation, both of which may be a function of local atomic order. Erickson and Roberts (*Phys. Rev.*, **B9**, 9; 1974) have measured the change on ordering in the electron charge density at the gold nuclei, and in the vibrational motion of the gold atoms in Cu_3Au.

Drijver *et al.* (*J. Phys., Paris*, **35**, C6–465; 1974) have used an analysis of the hyperfine fields in Ni_3Fe to determine the long range order parameter, and more recently (*Phys. Rev. Lett.*, **34**, 16; 1975) have extended this technique to follow the time evolution of the phase transition in this alloy 'at temperature'. A detailed analysis of the Mössbauer lineshapes confirmed that the transition was first order, proceeding by way of a nucleation and growth mechanism.

In this latest work, a sharp drop in mean field $\bar{H}_{hf}$ at the iron sites at about 771 K on heating indicates a first order transition, while during cooling, the reverse path is followed with a hysteresis of about 10 K. The linewidths (Γ) at the transition were much greater than the theoretical value (0.20 mm s^{-1}), mainly due to the presence of both ordered and disordered regions having values of H_{hf} which differed by a small amount (≈20 koersted). The amount of ordered material at 770.9 K roughly follows the increase in the mean field $\bar{H}_{hf}$ with time, along an S-shaped curve, consistent with nucleation and growth, taking about 200 hours to complete. By comparison with model calculated spectra, it was suggested that: (1) during the ordering reaction the first nuclei present have a long range order parameter of only about 0.6. There is then a homogenous growth of order within these ordered segregates. (2) During the disordering reaction there is in addition composition segregation involving iron-rich ordered material and iron-poor disordered material consistent with a two-phase region about 4 K wide similar to that observed by Calvayrac *et al.* (*Mater. Res. Bull.*, **7**, 891; 1972) using electron microscopy. While disordering starts at a temperature at or above the two-phase region, ordering is suppressed as shown by the hysteresis and starts about 10 K below.

The disadvantage of the Mössbauer technique is that for detailed measurements above room temperature it is really restricted to iron and possibly tin alloys, since for most other Mössbauer nuclides the size of the Mössbauer effect is too small. In the case of Ni_3Fe the amount of information available from the spectra is limited by the relatively small difference in H_{hf} between the ordered and disordered phase. One might expect a larger difference when a change in crystal structure is also involved. The use of small amounts of the Mössbauer nuclide ^{119}Sn as a probe in magnetic alloys might lead to a greater change in H_{hf}, since the tin atoms carry no magnetic moment and derive their field entirely from their neighbours. Thus the Mössbauer technique is restricted in its application in this field. But the advantage of being able to measure the changes in local properties at the temperatures concerned is considerable, and this technique, combined ideally with electron microscopy, could add greatly to our knowledge of order–disorder transitions in alloys.

Analysis of membrane noise

from Shin-Ho Chung

A NUMBER of researchers are using a powerful new technique to study electrical properties of nerve and muscle membranes. This technique, known as membrane noise analysis, is based on the fact that spontaneous perturbations of the mechanisms underlying membrane permeability give rise to random fluctuations in membrane conductance, and the statistical structure of these fluctuations provides, in turn, information about the microscopic events generating such noise.

On the basis of physical theory, electrical noise arising from several different sources is expected to be present in nerve and muscle membranes. First, the thermal agitation of charge carriers will produce thermal, or Nyquist, noise. In addition, when the flow of current is due to only a relatively small number of the total available carriers (as it is in the membrane), flicker noise is shown to be present. Superimposed on these two sources, there will be random fluctuations of membrane conductance resulting from microscopic perturbation of ionic channels. For example, single ionic channels in a membrane at an equilibrium state may spontaneously open and close, although the average number of open channels remains constant. The membrane conductance will then fluctuate around its mean value. Because each of these noise sources has a different physical basis, their analysis, in principle at least, will provide answers to different sets of questions which have not previously been amenable to experimental attack. An experiment by Conti, De Felice and Wanke (*J. Physiol., Lond.*, **248**, 45–82; 1975) was aimed at elucidating some of these questions.

Two physical models for ionic channels follow from Hodgkin and Huxley's description of current flows in membranes. The first of these, a probabilistic version of the Hodgkin–Huxley equations, envisages a channel gate, guarded by four macromolecules, either in an open or closed conformation. The channel is open when all these macromolecules are in their open conformation. An alternative class of

models, based on a literal interpretation of the Hodgkin–Huxley equations, predicts that channel conduction can occur even if the molecules of the gate are not all in the open conformation This condition allows the channel conduction to have more than just two (open and closed) states; rather, perhaps, a sufficient number of intermediate states to approach a continuum. Hill and Chen (*Biophys. J.*, **12**, 948, 1972, *ibid.* **13**, 1276; 1973) and Stevens (*Biophys. J.*, **12**, 1028; 1972) have independently derived that the spectral density for conduction fluctuations in the Hodgkin–Huxley axon (obtained from a Fourier analysis of the experimental records) obeys a mathematical form which, though not identical, resembles a simple Lorentzian function. The predicted spectrum is constant in the low frequencies and decreases according to $1/f^2$ in the high frequency limit. This holds true for both physical interpretations of the Hodgkin–Huxley equations, but the frequency at which the extrapolated $1/f^2$ decline intersects the low frequency limit is different in the two models.

Earlier measurements of voltage and current fluctuations in nerve membranes (Verveen and Dirksen, *Proc. IEEE*, **56**, 906; 1968; Poussart, *Biophys J*, **11**, 211; 1971) were dominated by the presence of flicker noise, and the most interesting noise, namely, that arising from ionic channels, went unnoticed. The expected Lorentzian noise from the squid axon was first detected by Fishman (*Proc. natn. Acad. Sci. U S.A.*, **70**, 876; 1973; *Fedn Proc.*, **34**, 1330, 1975) in his power spectrum of membrane voltage fluctuations. When tetraethylammonium ions (which are known to block potassium channels) were added to the bathing solution, the Lorentzian component disappeared, thus indicating that the detected noise arose from microscopic events associated with potassium permeability Because the voltage noise is related to conductance fluctuations by the membrane impedance, the elementary processes of nerve excitation can be revealed more directly through measurements of current noise. Conti *et al.* (also De Fecile *et al.*, *Fedn Proc.*, **34**, 1338; 1975) have now obtained two Lorentzian components, associated with the open–close kinetics of potassium and sodium channels (see figure). From the measurements, they also derived the number of sodium and potassium channels present per unit membrane, and, from it, they calculated the absolute conductance of individual channels. Their estimate of the number of sodium channels, 330 per square micrometre, agrees well with those derived from other lines of argument (see Hodgkin, *Phil. Trans. R. Soc.*, **B270**, 297; 1975; Keynes *et al.*, *ibid.*, 365). Available data, however, are not refined enough to distinguish between the two possible physical interpretations of the Hodgkin–Huxley equations, but with luck they soon will be, as a number of laboratories are now making measurements of axon conductance fluctuations.
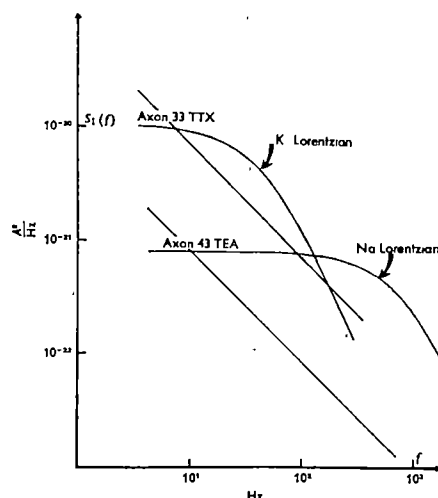
Components of current noise spectral density from squid axons When sodium channels were blocked with tetrodotoxin, the spectrum consisted of the two separate components (flicker + K Lorentzian). Similarly, in an axon with its potassium channels blocked, the sum of the two components (flicker + Na Lorentzian) could adequately describe the obtained spectrum. (Modified from Conti *et al.*, *J. Physiol*, *Lond.*, **248**, 45; 1975.)

Analysis of noise in the muscle membrane has also provided important sets of data. One of the possible physical models emerging from such measurements is that two acetylcholine molecules binding on a receptor induce a conformational change in a gating macromolecule (Stevens, *Fedn Proc.*, **34**, 1364; 1975). The channel then allows the transmembrane flux of sodium and potassium ions The length of time a channel stays open depends exponentially on membrane potential; for each -100 mV change in membrane potential, the lifetime of open channels doubles. At $8°C$ and 0 mV, for instance, a channel remains open, with a conductance of 20 to 30 $p\Omega^{-1}$, for about 6.5 ms. These conclusions could have been derived directly, were it possible to activate a known number of channels by iontophoretically applying acetylcholine at the endplate and, instantaneously, to cause the relaxation of the receptors back to their normal, closed, conformation. Because such an experiment is not technically feasible, quantitative information such as that obtained by Stevens can only be acquired by membrane noise analysis.

Katz and Miledi (*Nature*, **226**, 962; 1970) first discovered that a constant dose of acetylcholine applied to the frog neuromuscular junction gives rise to a substantial increase in membrane noise. In a subsequent analysis (*Nature new Biol.*, **232**, 124; 1971; *J. Physiol.*, *Lond.*, **224**, 665; 1972), they showed that this increased noise results from endplate gating mechanisms, and calculated the size and time course of the elementary event. The technique of noise analysis was further exploited by Anderson and Stevens (*J. Physiol.*, *Lond.*, **235**, 655; 1973) Making the assumption that the receptor molecule exists either in open or closed conformation, Stevens and his associates (see also Magleby and Stevens, *J. Physiol*, *Lond.*, **223**, 151; 1971; *ibid.*, 173) made a number of predictions about the behaviour of conductance fluctuations, making use of the fluctuation-dissipation theorem The measurements of acetylcholine-induced conductance fluctuations, obtained under voltage clamp conditions, agreed well with the theoretical predictions. The striking agreement between theory and experiment does not, however, constitute final proof of the theory proposed by Stevens. In fact, a slightly different version of microscopic events, in which a channel opens instantaneously and its conductance then decreases exponentially (Katz and Miledi, 1971), will lead to the same prediction about the behaviour of conductance fluctuations. There is, furthermore, an additional observation, for which Stevens's model. in its simplest form. cannot account without making additional assumptions. This is the observation that the mean lifetime in the open state, and the absolute conductance of a single channel, can each vary when different drugs are used to open the channel (Katz and Miledi, *J. Physiol.*, *Lond.*, **230**, 707: 1973; Colquhoun *et al*. *Nature*, **253**, 204; 1975). The model would have predicted that a channel, once it is open. will have the same conductance whichever drug caused it to open. Nevertheless. theoretical predictions of the type Stevens has made, along with the quantitative data extracted from membrane noise analysis, will place a severe constraint on a number of tenable physical models for endplate gating mechanisms.

In the closing chapter of his classic book. *Membranes. Ions and Impulses* (University of California, Berkeley, 1968), Cole suggested that a sequel of his volume should be written in a decade or so under the title of *Membranes, Ions and Impulses: A Chapter of Molecular Biophysics*. When that chapter is finally written, we will have witnessed the final act of a long drama, staged by some of the intellectual giants

of the past and present It is toward this goal that a group of neurophysiologists are making steady progress; and analysis of membrane noise is but one of the many tools at their disposal.

Ordered disorder?

from P. V. E McClintock

A NEW theory of the dilute magnetic alloys known as spin glasses has been proposed by Edwards and Anderson. The burden of their argument, published in *J. Phys.* (**F5**, 965; 1975), is that an apparently random arrangement of magnetic spins may, in reality, sometimes be regarded as being highly ordered.

An example of the type of alloy to which their theory applies is formed when a few per cent of manganese is added to copper: the copper, being non-magnetic, serves as a relatively inert medium in which the manganese atoms are held fixed in random spatial positions. The unpaired electron spins responsible for their magnetism are, however, free to point in different directions. At high temperatures the spins will be randomly orientated with their directions changing with time, but the intriguing question arises· what will happen as the temperature is reduced?

In order to try and guess the answer it is instructive, first, to consider what happens in a pure magnetic material, for example pure manganese, where the spins are all equally spaced from each other In such a case the mutual interactions between nearest neighbour pairs of spins would all be the same, and the system would either become ferromagnetic (all spins pointing in the same direction) or antiferromagnetic (neighbouring spins pointing in opposite directions). Which of these two types of ordered state is the one actually adopted is a sensitive function of the spacing between the atoms Measurements of the magnetic susceptibility χ as the temperature T is reduced show that the change from the disordered to the ordered state occurs as a so-called cooperative transition, with $\chi(T)$ passing through a sharp anomaly at a critical temperature T_o. This behaviour can be understood if one postulates a critical temperature T_c. This behaviour spins: the larger the number of ordered spins, the larger the molecular field becomes, thus accounting for the sharpness of the anomaly and also, of course, for the description of the transition as cooperative.

On the other hand, when the manganese is diluted with copper, the magnetic atoms will no longer be spaced equally from each other, and the whole situation becomes very much more complicated. The simple molecular field approach is no longer applicable because some pairs of spins will be attempting to align while others, with different mutual separations, will be tending to take up antiparallel orientations. Moreover, the different spin spacings will tend to give rise to different values of the temperature T_o at which the ordering transition occurs. Thus, common sense would seem to suggest that any peak in $\chi(T)$ must be greatly broadened. Experiments, however, have demonstrated quite otherwise: Canella and Mydosh (*Phys Rev.*, **B6**, 4220; 1972) for example, working on the somewhat similar iron–gold system, found that for an alloy containing 5% of iron there was a very sharp cusp in $\chi(T)$ at about 20 K.

To try to account for this, Edwards and Anderson have pointed out that, despite their random positioning, there must be some configuration of the orientations of the spins which minimises their potential energy. Considering the spins' positions in space to be fixed and permanent, this configuration can then properly be regarded as the ground state of the system (in fact, one of many such states, each corresponding to a local minimum in potential energy). The authors argue that the mere existence of such a state is sufficient to give rise to a well-defined magnetic ordering transition, and hence to account for the observed sharp cusp in $\chi(T)$. Thus, as T is reduced, there must eventually come a temperature at which the spins 'notice the existence' of this ground state; and, as $T \rightarrow 0$, the system will then settle into that state. Of course, if one were able to examine the material in its ground state, it would seem, superficially at least, still to be in total disarray with the spins arranged quite randomly. The randomness would be more apparent than real, however, because the system would be in the particular state which minimised its potential energy. It thus could reasonably be regarded as being, in actual fact, highly ordered.

These ideas are by no means original, other workers having previously arrived at broadly similar conclusions. The great contribution which Edwards and Anderson make in this paper is that of devising a theoretical framework within which the magnetic behaviour of such systems may be treated quantitatively. They describe the degree of order in the spin system by a parameter q which represents the probability that a spin pointing in a particular direction at one moment will be pointing in that same direction when viewed again a long time later. Thus at $T=0$, where the system is magnetically in a state of perfect order, $q=1$; while for $T \geqslant T_o$, there is no long-range order, and $q=0$. The order parameter q therefore be-

haves rather like the molecular field of the familiar Curie–Weiss theory. The authors have, in fact, worked out these ideas at a similar level of accuracy to that of the Curie–Weiss theory, and they suspect that their conclusions must be inaccurate in similar ways, notably in a wrong prediction of the detailed shape of the cusp in $\chi(T)$· they are generously leaving the application of quantum mechanical refinements to other workers.

Their main conclusion, however, is the important one: that, even though the alloy may be neither ferromagnetic nor antiferromagnetic, it will nevertheless undergo a magnetic ordering transition which should manifest itself as sharp peaks in properties such as the magnetic susceptibility, and at a temperature which can in principle be predicted. This is a remarkable theoretical result, but one which is in excellent accord with reality as revealed by the experiments.

Mutating and mapping SV40

from Lois K. Miller

NUCLEIC acid biochemists are now able to manipulate the DNA genome of small animal tumour viruses with remarkable skill as illustrated by two papers from Paul Berg's laboratory (Shenk *et al*, *Proc. natn Acad. Sci. U.S A*, **72**, 989, 1975; and Carbon *et al*, *Proc. natn. Acad. Sci U.S.A.*, **72**, 1392, 1975). These workers have exploited the known specificities of several types of DNA nuclease to produce viral deletion mutants and map those mutants with astounding ease, accuracy, and rapidity.

Mutations in small animal viruses are difficult to map by the more conventional genetic methods because of their size, low recombination frequencies, and lack of different mutant types for three-factor genetic crosses. A technique, originally developed for ΦX174 (Weisbeek *et al*, *Biochim. Biophys. Acta*, **224**, 328; 1970; Hutchison and Edgell, *J Virol.*, **8**, 181; 1971), was modified by Lai and Nathans (*Virology*, **60**, 466; 1974), and utilised to map temperature sensitive (ts) mutations of SV40. The technique relies on the ability of wild-type DNA fragments to rescue a mutant DNA genome when the two are properly annealed together and introduced to an appropriate cell. When wild-type restriction endonuclease fragments are employed, the mutation can be localised within a single fragment and the relative position within the genome is known since restriction nuclease fragments can be ordered by independent means. Al-

though it is known from the ΦX174 studies that fragments 30–50 nucleotides long can rescue mutant genomes, the achievement of such accuracy in mapping with this technique would require a tour de force on the part of the investigator Nevertheless, marker rescue is a rapid direct approach to mapping ts mutations within a region of the genome 300–500 nucleotides in length.

Shenk *et al*. have developed a purely biochemical mapping technique which is based on the ability of S_1 nuclease, an endonuclease from *Aspergillus oryzae* which recognises regions of single-stranded DNA, to produce double-stranded cleavages at the mismatched region of a wild type–mutant heteroduplex The technique is ideal for mapping small deletion mutations (too small to map by electron microscopy heteroduplex techniques) due to its rapidity and accuracy. Although Shenk *et al*. have also mapped ts mutations using this technique, it is apparent from their results that single-base mutations are not easily recognised by the S_1 activity. In mapping ts mutations, Shenk *et al*. observe very high backgrounds due to non-specific cleavage of the DNA and the 'nibbling' of the breathing ends of the double-stranded DNA by the S_1 nuclease. It would be interesting to scan the presently known repair endonucleases for an enzyme with higher specificity for single-base mismatched regions and couple such an enzyme with the S_1 nuclease technique.

There are several advantages the S_1 mapping technique has over the marker rescue technique. The S_1 technique, according to Shenk *et al*., is accurate to 1% of the SV40 genome or approximately 55 nucleotides and thus gives ten times more accuracy with approximately equal ease. Since the S_1 technique is purely biochemical, it does not rely on infectivity and is potentially useful for mapping non-viral DNA (including eukaryotic DNA) or viral DNAs having low plaque forming units/µg DNA ratios (for example adenovirus 2). But since it is purely biochemical, caution must be taken that a second-site, silent mutation is not mistaken as the true mutation.

Carbon *et al*. have developed an excellent technique for producing deletion mutants of SV40. By digesting 25 to 30 nucleotides from the ends of a linear double-stranded DNA with the 5′-end specific lambda exonuclease, they produce shortened DNAs which, when introduced into monkey cells, yield circular deleted genomes in a manner similar to that previously reported by Lai and Nathans (*J. molec. Biol*., **89**, 179; 1974) at a high frequency. By using appropriate combinations of DNA nucleases, it is

apparent that Carbon *et al*. will be able to produce deletion mutants not only at restriction nuclease sites but also at essentially random sites throughout the SV40 genome. Since these deletion mutations can be mapped readily by the procedure of Shenk *et al*., such mutants should be most helpful in defining essential regions of the genome and the limits of the various genes.

Previous reports on isolating deletion mutants (Mertz and Berg, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4879; 1974); Lai and Nathans, *J molec. Biol*.) have shown that certain deletion mutants are viable whereas other mutants are non-viable and must be carried with helper virus. The latter case presents problems in obtaining pure stocks of deletion mutants. It would be useful in this respect to develop stocks of helper virus which could contribute functions lacking in the non-viable deletion mutants yet be easily separable from the deletion mutant itself. It would also be useful to develop *in vitro* techniques for the production of specific conditional lethal mutants (such as ts mutants) at high frequencies.

Lipid
on the run

from A G. Lee

THE nightmare of anyone using probe molecules to study membranes is of awakening to find that all that has in fact been studied is the probe. Hence the tendency to seek confirmation of previous results using new probes and new physical techniques, on the principle that a nightmare shared is a nightmare halved.

The latest example of this concerns the measurement of the diffusion rates of lipids within the plane of a lipid bilayer These measurements have generally been based on studies of the magnetic interactions between molecules in the membrane. If a spin-labelled steroid or lipid is incorporated into the membrane at high concentrations, then magnetic interactions occur between the unpaired electrons of the spin-labelled molecules, and this interaction is reflected in the shape of the electron spin resonance spectrum. If the spin labels are moving in the plane of the membrane, then the movement will modulate the magnetic interactions, the exact effect of the motion on the shape of the spectrum depending on the rate of the motion. In this way, Träuble and Sackmann were able to estimate a rate of lateral diffusion for spin-labelled androstan of about $1-2 \times 10^{-8}$ cm^2 s^{-1} (*J Am. Chem. Soc*., **94**, 4499; 1972). Devaux and McConnell

(*J. Am. Chem. Soc*., **94**, 4475; 1972) obtained similar results, also with spin-labelled molecules, but using a kinetic technique to detect diffusion. A diffusion rate of this magnitude is of considerable interest since if the lipids in a real biological membrane were moving as fast, it would mean that a lipid could move from one end of a 1 µm long bacterium to the other in the order of a second.

An alternative approach was to look at the magnetic interactions between the protons of the lipid fatty acid chains which are reflected in the proton nuclear magnetic resonance line shapes. This has the advantage that it is possible to use natural phospholipids, with no perturbing spin-label, but it has the disadvantage that it is rather difficult to separate contributions to the linewidth from the diffusive motion and from other sources. However, the self-diffusion coefficients obtained in this way were identical to those obtained by electron spin resonance (Lee *et al*, *Biochemistry*, **12**, 1650; 1973).

In the latest paper (*Proc natn. Acad. Sci. U S A.*, **72**, 1451; 1975) on lateral diffusion Brulet and McConnell have extended the theory of time-dependent magnetic interactions to the case of two-dimensional diffusion This has enabled them to look in detail at the effect that a spin-labelled molecule incorporated into a lipid bilayer will have on the relaxation times of ^{13}C or ^{1}H nuclei of the lipids of the bilayer. Unfortunately, for the calculations to be at all tractable it was necessary to assume that the two-dimensional lateral diffusion of the spin label was confined to one single plane, at some fixed depth within the lipid bilayer. Similarly it had to be assumed that the nucleus of interest was also forced to diffuse laterally within a fixed plane. Neither of these assumptions is strictly valid, since it is known that phospholipids undergo extensive vibrational motion, so that positions in a direction perpendicular to the bilayer surface are constantly changing. Although it is not known what effect this will have on the results, it is more than a little comforting that what emerges at the end of the calculations is once again the magic number obtained by the other techniques—2×10^{-8} cm^2 s^{-1}.

It now seems very unlikely that these results for lipids in simple lipid bilayers will be overturned. When we turn to the intact biological membrane, however, an interesting problem emerges. If all the lipids in biological membranes were to have such high diffusion rates, then there could be no persistent interactions between specific proteins and lipids in the membrane, and the environment of the membrane proteins would be a weighted average of that provided by the heterogenous mixture

Superconducting regions in lysozyme

from a Correspondent

GREAT interest was aroused at the Dielectrics Society annual meeting by Herbert Fröhlich's report of some experiments which show that an aqueous solution of the enzyme lysozyme contains regions which are superconducting. The report (now published: Ahmed, Calderwood, Fröhlich and Smith, *Phys. Lett.*, **53A**, 129; 1975) can be summarised thus.

Magnetic fields of order 600 Gauss were found to bring about very large changes in the dielectric constant of aqueous lysozyme solutions. Since for this to happen the magnetic field must be able to overcome ionic thermal fluctuations, the magnetic energy must act over a volume of the order of a million lysozyme molecules, which indicates the existence of a cooperative phenomenon capable of increasing the magnetic susceptibility in a

magnetic field.

This measurement was therefore made, and a 1% increment in bulk susceptibility was found on application of the 600 Gauss field. This is ten thousand times larger than the effect for an ordinary diamagnetic material and its disappearance about 800 Gauss indicates that it represents a Meissner effect in superconduction. Complicated temperature and concentration effects were found, including temperature hysteresis. It was postulated that in solution the ions form a layer structure, giving rise to an a.c. Josephson effect, that is, to the electric vibrations in biomolecules recently proposed by Fröhlich and reported in the Soviet literature. Physiological effects of magnetic fields would seem to be given physical respectability if a Meissner effect exists in a biomolecular solution.

---

of chemically distinct lipids present in that membrane. That goes against the considerable weight of experimental evidence suggesting specific interactions between particular proteins and lipid classes, with at least a ring of lipid bound relatively tightly to the protein in a long-lived interaction. So it seems that whereas the bulk of the lipid could be diffusing as fast as lipid in simple lipid bilayers, there might also be some lipid in contact with protein and exchanging only slowly with the bulk lipid.

In a recent paper, Warren *et al.* (*Nature*, **255**, 684; 1975) explore this possibility of relatively long-lived lipid–protein interactions with their simplified lipid–protein system, containing the (Ca^{2+}–Mg^{2+}) ATPase from sarcoplasmic reticulum. They have found that the activity of the protein is maintained as long as there are 30 or more phospholipid molecules for each protein molecule: with a smaller proportion of phospholipid there is an irreversible loss of activity. It has been suggested therefore that these 30 lipid molecules form a single shell of phospholipid bilayer around the protein, protecting it from denaturation. Further experiments have then shown that cholesterol, which on its own leads to a reversible loss of activity, cannot normally penetrate this lipid annulus. If, as seems likely, this proves to be a general property of membrane proteins, then the organisation of lipids within the membrane will be by no mean homogeneous. Each protein will be surounded by a lipid microenvironment, the structure of the

protein and the nature of the microenvironment together determining the functioning of the protein. Only the bulk lipid will be free to diffuse within the membrane, and around each protein there will be an immobilised lipid annulus, whose composition will be determined by the structure of the protein. Even in the transitory world of membranology, not everything is in a state of flux.

Mammalian development, with chicks and newts

from Gillian M. Morriss

The Ciba Foundation held a symposium on "Embryogenesis in Mammals" on June 16–19. The proceedings will be published by Associated Scientific Publishers.

THE symposium was held just ten years after the symposium on preimplantation states of pregnancy—ten years which have seen a tremendous creative surge in the field of vertebrate experimental embryology. With congenital anomalies an increasing medical problem, much of this creative energy has been applied to studying normal and abnormal postimplantation mammalian development. Whole embryo and organ culture techniques have contributed ~atly to the accessibility and ma⁣ ⁣ity of the systems under

investigation; however, in comparison with the work on other vertebrates, mammalian embryology appears to have embarked on an obstacle course beset with many frustrations. Thus it was fitting that two presentations on recent advances in avian and amphibian development were included in the symposium, and that four additional non-mammalian embryologists were present as discussants.

Death of a germ layer

More than one established dogma was challenged by the participants. The first was the demonstration by R. L. Gardner (University of Oxford) that the early differentiation of inner cell mass into 'endoderm' (hypoblast) and 'ectoderm' (epiblast) does not signify the establishment of cell lineages loyal to the historical concept of germ layers. The most important determinative factor in the differentiation of preimplantation and implanting embryos is cell position; thus chimaeras can be constructed whose primary endoderm-derived cells are detectably different from the rest of the embryo. Examination of such embryos at 15½ days showed that the whole embryo is derived from the so-called ectoderm. Other experiments demonstrated that even the germ cells are ectodermal in origin.

N. Skreb (Faculty of Medicine, Zagreb), describing a study carried out in collaboration with B. Levak-Svajger and A. Svajger (see *J. Embryol. exp. Morph.*, **32**, 445–467; 1975), showed that, when transplanted to a site under the adult kidney capsule, embryonic ectoderm retains the capacity to differentiate into all embryonic structures (including gut) up to the primitive streak stage, though not later. Endoderm alone was always resorbed, but this may simply be a further demonstration of the essential interactive role of adjacent mesenchyme and intercellular matrix in epithelial differentiation. Nicole Le Douarin (Université de Nantes), for instance, showed that grafts of portions of avian pharyngeal endoderm will not differentiate in the absence of mesoderm. The major part of her paper concerned that unique piece of ectoderm, the neural crest. Her elegant studies on quail–chick transfers are too well known to need description here (see, for instance, *Devl Biol.*, **41**, 162–184; 1975); as the chairman, Anne McLaren (University College, London) remarked, they are a source of envy to mammalian embryologists. A further characteristic of the primitive endoderm is its slow proliferation: M. Snow (University College, London) calculated that in the primitive streak stage mouse embryo, the cell cycle time in the endoderm is more than 15 h, compared to 6½ and

8 h in the other two layers.

The question of the developmental capacity of the primitive (or primary) endoderm is clearly as yet unresolved. But does the term 'germ layer' remain meaningful or useful? Small wonder that by the end of the symposium there was general agreement that the products of the first differentiation of the inner cell mass be designated 'epiblast' and 'hypoblast', in common with avian terminology.

A day of consideration of genetic aspects began with the questions: "How early do genes begin to function in mouse embryos?" and "Is there a genetic programme for the timing of gene expression?" Using β-glucuronidase as a specific product of gene expression in two strains of mice whose rate of synthesis of the enzyme differs, V. M. Chapman (Roswell Park Memorial Institute, Buffalo) suggested that the gene involved is expressed as early as the two-cell stage.

Postimplantation developmental abnormalities were described in a variety of trisomies in mice by A. Gropp (Lubeck Medical School). Like human trisomies, these are all associated with abnormal facial morphogenesis, but the mechanisms are unknown.

Gene action in abnormal preimplantation development of mouse embryos homozygous for the yellow allele of the agouti locus was demonstrated by R. A. Pedersen (University of California, San Francisco) by means of timelapse cinemicrography of cultured embryos. The abnormalities culminating in preimplantation loss consisted of delayed or arrested cell division during cleavage stages, and failure of extension and adhesion of trophoblast cells to the culture dish at the implantation period. One may speculate that the lethal gene affects the synthesis of one of the proteins which are involved in both spindle formation and cell motility or adhesion.

Abnormalities of cell motility and cell–cell interaction are characteristics of abnormal gene expression in T-locus mutant mice. In an ultrastructural study of the lethal t^9-homozygote, Martha Spiegelman (Cornell University Medical College) showed that newly formed primary mesenchyme cells lack subsurface microfilaments, do not make organised cell contacts, and fail to migrate away from the primitive streak. In T-homozygotes the abnormality involves defective development of the basement lamina of the neuroepithelial cells, which are consequently able to make close associations with underlying notochord and somite cells. Thus alterations in the cell surface are of major importance in the changing interactions between cell and environment. In this context, M.

Edidin (Johns Hopkins University) examined three types of antigen, and changes in the concanavalin A binding pattern, as indicators of alterations in cell surface components. The results confirmed that new antigens are expressed during the course of differentiation, and that their quantity alters. Con A receptors come and go on mouse embryonic cells: they increase up to the blastocyst stage, but in the blastocyst itself they disappear from trophoblast cells and are localised exclusively in the inner cell mass.

W. J. Rutter (University of California, San Francisco) showed that actual cell contact may not be necessary for differentiation of the pancreas. The epithelium will develop its characteristic glandular structure when in contact with sepharose beads coated with a factor derived from mesenchyme. The cell surface receptors influence the synthetic abilities of the cell, under appropriate external stimulation. In the context of the cell–cell interactions, the cell surface must be taken to include the surface coat. This was clearly demonstrated by R. O. Kelley (University of New Mexico, Albuquerque), using the epithelial–mesenchymal interaction during human limb morphogenesis as a model system, and an impressive armoury of techniques. The mesenchymal cells have both receptor and catalytic components of the adenylate cyclase system, and the receptivity of this system to exogenous stimulation is potentiated by diminishing or removing the cell surface glycosaminoglycans (GAGs). The fact that trypsin reduces the thickness of cell surface GAGs should be borne in mind by all experimenters using this enzyme.

Although the stability of the differentiated state is still acceptable as a general rule, studies on transdifferentiation (well established in connection with regeneration in amphibia) have now been extended to avian embryos. G. Eguchi (University of Kyoto) showed that in the case of both chick and newt, cloned pigmented retinal epithelial cells will give rise to a few lens cells. The time period is about 90 days in the chick compared with less than 40 in the newt, but the process can be speeded up, *in vivo* as well as *in vitro*, by the use of a small crystal of nitrosoguanidine (a potent mutagen/carcinogen). The outcome of similar experiments on mammalian cell lines is awaited with interest.

Those present at the symposium spanned a great many of the component areas of this exciting field of research. The discussions were both wide-ranging and controversial. Such a stimulating symposium can only add to the accelerating impetus which all areas of developmental biology are undergoing at the present time.

A comet in the Plough

from Robin Scagell
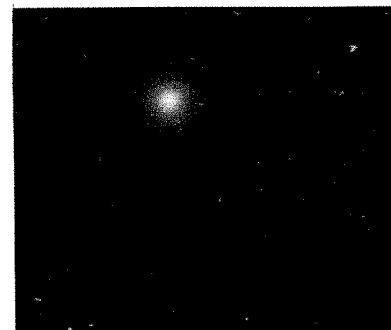
THE first comparatively bright comet to appear since the ill-starred Kohoutek is in the evening sky at the moment, and is making up for its predecessor's fickleness by being readily observable with binoculars. It could well provide an interesting sight within the next few weeks.

Comet Kobayashi–Berger–Milon, named after its three independent discoverers, was first spotted from Japan on July 2, and has the designation 1975h. It has been moving through the evening sky towards the Sun, to which it makes its closest approach on September 5. Much to the delight of those who spent cold and fruitless vigils trying to glimpse Comet Kohoutek, 1975h has passed almost through the zenith for observers in Britain and America and was easily seen even on nights of full Moon. By July 24, although no tail was noticeable, the object appeared as a fairly large circular diffuse patch of light of about fifth magnitude—just visible to the naked eye.

Kobayashi–Berger–Milon represents a good run of the mill comet which is worth following. It has even had the good sense to appear to mingle with the stars of that best known of all northern constellations, Ursa Major—the Plough or Big Dipper. At the beginning of August it will pass close to the well known double star Mizar, the middle star in the handle of the Plough or Dipper, and should be easily visible with even the smallest pair of binoculars.

Throughout August it will move steadily south-eastwards until it reaches perihelion on September 5 in Leo Minor, where it will best be seen in northerly latitudes at twilight. After perihelion it will become less readily visible.

articles

Three-dimensional structure, function and genetic control of immunoglobulins

Roberto J. Poljak*

Recent crystallographic analyses of the three-dimensional structure of immunoglobulins have shown how it is related to their binding affinities. Considered with evidence from sequencing, it is beginning to cast some light on how the genetics of immunoglobulins could have evolved to maximise binding diversity with a minimal genetic load.

SERUM antibodies are glycoproteins generically denominated immunoglobulins. Although in recent years much progress has been made in analysing experimentally induced antibodies[1], the structure of immunoglobulins has been most intensively studied using human and murine myeloma proteins which provide homogeneous material in the quantity required for structural analysis. It is now widely accepted that antibodies and myeloma immunoglobulins are closely related molecules. The antibody response against a specific antigen (or hapten) is usually degenerate, consisting of a heterogeneous population of molecules, a fact which complicates structural analyses. In contrast, myeloma immunoglobulins are homogeneous but the elucidation of their hapten binding specificity may require an intensive search.

Several classes (or isotypes) of immunoglobulins (Ig) are present in normal or pathological sera and of these the IgG (or γ globulin) class is the most abundant and has been most intensively studied. A diagrammatic representation of a human IgG immunoglobulin is shown in Fig. 1. The comparison of the amino acid sequences of internal segments of the heavy (H) and light (L) polypeptide chains have led to the recognition of homology regions about 110 amino acid residues long[2]. The homology region designated as C_L (Fig. 1) is constant in amino acid sequence and characteristic of a given subclass (κ or λ) of L chains which are present in all immunoglobulin classes. C_H 1, 2, 3 are also constant in sequence and characteristic of a given class or isotype of immunoglobulin defined by its H chain. When the amino acid sequences of different immunoglobulins are compared, the regions designated as V_L, V_H (Fig. 1) show variability, in particular around positions 25–30, 50 and 95–100 in V_L and 30, 55–60 and 105–110 in V_H, positions which are called "hypervariable"[3]. These hypervariable regions have been postulated by many workers to be the determinants of the conformation and specificity ("complementarity") of an antibody combining site, and to be closely related to the "idiotypic"[4,5] antigenic markers which are unique to immunoglobulins secreted by a clone of cells.

Three-dimensional structure of immunoglobulins

Models of Fab' New obtained by X-ray crystallographic analyses at 6 Å, (ref. 6), 2.8 Å (ref. 7) and 2.0 Å (ref. 8) resolution showed that Fab' consists of two globular domains, V and C, separated by an internal space accessible to solvent. Each domain consists of two globular subunits corresponding to the V_L and V_H homology regions in the V domain, and to C_L and C_H1 in the C domain. V_L and C_L as well as V_H and C_H1 are

joined by narrow, linear regions by which the L and H polypeptide chains cross from the V to the C domain. These regions are exposed, accessible to solvent and to attack by agents such as proteolytic enzymes. The overall structure is that of four "homology subunits" in tetrahedral arrangement. In agreement with their sequence homology and the ensuing postulate that they arose from a common ancestral gene[2], V_L, V_H, C_L and C_H1 share a basic pattern of polypeptide chain folding although V_L and V_H include an additional loop of polypeptide chain (Fig. 2). In each homology subunit two irregular, roughly parallel β sheets consisting of strands of antiparallel polypeptide chain surround a tightly packed interior of hydrophobic side chains including the intrachain disulphide bond. In the variable homology subunits V_L and V_H, the hydrophobic interior consists of invariant or semi-invariant amino acid side chains. The same overall pattern of polypeptide chain folding was observed in the 3.5-Å resolution structure of the Mcg L-chain dimer[9]. Different arrangements of interchain disulphide bonds which occur in other immunoglobulin molecules could be explained on the basis of the Fab' New model. Since these different disulphide bonds occur in macroglobulins (IgM), in
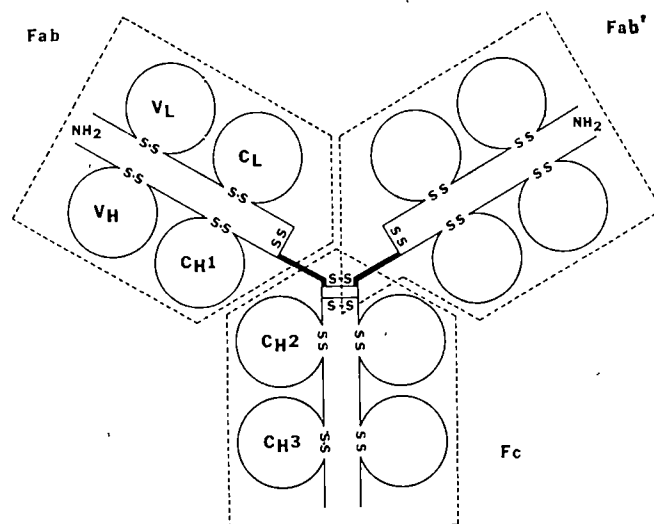
Fig. 1 Diagrammatic structure of a human IgG1 molecule. The light chains are divided into two homology regions, V_L and C_L. The thicker lines in the heavy chains correspond to the "hinge" region. The four homology regions (V_H, C_H1, C_H2, and C_H3) of the heavy chains, the interchain and intrachain disulphide bonds, the N-terminal region of both chains, and the major fragments (Fab, Fab', Fc) are indicated. Reproduced from Poljak[33].

*Department of Biophysics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.

secretory immunoglobulins (IgA) and in other immunoglobulin isotypes, it was postulated[7] that the basic immunoglobulin fold (Fig. 2) is present in all classes of immunoglobulins. This postulate was given substantial support by the results of the crystallographic analysis[10] of the Fab fragment from the McPC 603 IgA, a murine immunoglobulin secreted by cells of an experimentally induced mouse myeloma tumour. The H (α) and L (κ) polypeptide chains present in this immunoglobulin are of different isotype from the corresponding chains of the human Fab′ but the overall three-dimensional structure is similar. Further, the high resolution structure of the variable part of a human κ chain (a Vκ dimer) indicated the same folding of the polypeptide chain[11]. At the present stage of the structural analysis of immunoglobulins the postulate that they all share a common pattern of three-dimensional structure (Fig. 2) can be accepted with reasonable confidence. The "hinge" region connecting Fab to Fc is not included in this pattern; different isotypes of immunoglobulins differ in this region which determines a segmental flexibility[12]. Within one isotype of immunoglobulin, the region that determines the antigen combining site (or active centre) makes for variability in structure and function.

The combining site

The atomic resolution model of the human Fab′ fragment[7] showed that the hypervariable regions of both L and H chains occur at one end of the molecule, in spatial proximity and fully exposed to solvent with all the characteristics of a combining site. A similar observation was made in the structure of Fab from the McPC 603 IgA (ref. 10). Thus, different immunoglobulins will present a unique, idiotypic structure at this end of the molecule. In the L chain dimers Mcg[9] and Rei[11] there is a cavity surrounded by the hypervariable regions of both chains which resembles the combining site of the Fab fragments, although it is wider and symmetrical around a central twofold rotation axis. The combining site of IgG New consists of a shallow groove, about 15 × 6 Å, with a depth of about 6 Å. The active centre of McPC 603 is deeper (about 12 Å) than that observed in IgG New. This increased depth can be attributed in part to the fact that the first hypervariable region of the κ (L) chain of McPC 603 is six amino acids longer (by an "insertion" of the type shown in Fig. 3 for human Vκ_III sequences) than other murine and human κ and λ chains. This inserted sequence protrudes to form a "deeper" active site. It follows that the pattern of insertions and/or deletions, characteristic of the hypervariable regions (in particular around residue 105 in human H chains) will be the major determinant of the dimensions of the active site. Variations in sequence or amino acid replacements which are also a characteristic of those regions will determine the chemical environment of the site. Thus, different antibodies may display unique antigen complementarity sites although they share a common three-dimensional structure. A vast number of recognition specificities can be incorporated in this design, although a constant, compact immunoglobulin fold is maintained. A further consideration is that, since both H and L chains contribute to the combining site, random pairing could give rise to a large number of different combining sites (n^2) with a much smaller number (n) of H and L chains, further extending the economy of structural design. The number of germ line structural genes required to encode all necessary H and L chains in an immunologically mature animal would thus approach a reasonably small value ($n \leqslant 10^3$) when compared with the total number of structural genes in the genome of vertebrates. Evidence of recombination of H and L chains *in vitro* has been obtained in several laboratories (for reviews see refs 13 and 14); and the possibility that recombination is restricted to mutually compatible H and L chains has also been submitted to limited experimental tests[13,15]. Experimental tests of *in vitro* recombination of H and L chains would however have to cover all possible subgroup and isotype permutations (subgroup is used to designate a set of V_H or V_L sequences which are similar or more closely related to each

other than to those of another subgroup). A study of this problem by analysis of the interactions between V_L and V_H in the three-dimensional structure[16] reveals close interactions between amino acid side chains at positions 35, 37, 42, 43, 86 and 99 in V_L and at positions 37, 39, 43, 45, 47,95 and 108 in V_H. These positions are occupied by constant residues or by fairly conservative replacements in human V_L and V_H sequences as well as in V_L and V_H sequences that have been studied from other animal species. Furthermore the amino acid sequences of human κ and λ chains are very similar at these positions: almost all human L chains have Tyr (35), Gln (37), Ala (42), Pro (43), Tyr (86) and Phe (99). L chain positions 88 and 90 occur at the active site and also seem to provide for V_L–V_H interactions but the importance of these interactions is more difficult to assess because they could be affected to some degree by the conformation of the last hypervariable region of V_H; even so there are preferred residues at these positions: Gln (88) and Trp/Tyr (90). In the C_L region κ and λ chains share the sequence of residues which interact with C_H1. Thus, the interactions between V_H and V_L, which should provide the stereochemical basis for their association, seem to be independent of H and L chain subgroups or even L chain class. Although preferred associations could occur under genetic control there is no apparent structural basis for restricted pairing. Different H, L chain associations could be expressed under selective stimulation by antigens, thus making use of all the structural information contained in the genome.

Fig. 2 Diagramm of the basic 'immunoglobulin fold'. Solid trace shows the folding of the polypeptide chain in the constant subunits (C_L and C_H1). Numbers designate L (λ)-chain residues, beginning at "NH_3+" which corresponds to residue 110 for the L chain. Broken lines indicate the additional loop of polypeptide chain characteristic of the V_L and V_H subunits.

Fig. 3 Amino acid sequences of the N-terminal region of human κ chains arranged in subgroups, Vκ$_I$, Vκ$_{II}$ and Vκ$_{III}$. Reproduced from Gally and Edelman[34].

In human L chains, subgroups can be defined by (or correlated with) the presence of insertions (or deletions) in the first hypervariable region (Vκ, Vλ) and also in the third hypervariable region (Vλ). Consequently, these subgroups define different conformations of the active site, as discussed above. For example, human κ chains of subgroup III (Vκ$_{III}$, see Fig. 3) will delineate a "deeper" active site such as that observed in the murine myeloma protein McPC 603 (ref. 10) than will a κI or a λ chain. Since murine λ chains seem to lack the pattern of deletions and/or insertions at the hypervariable regions characteristic of murine κ and human κ and λ chains, they should be expected to make a smaller contribution to the diversity of antibodies. Thus the number of genes encoding λ chains or their expression in mice would be limited relative to κ chains as reflected in the κ/λ serum ratio[17].

Hapten binding, cross reaction and multispecificity

An increasing number of human and murine myeloma proteins has been shown to bind ligands in a reaction that closely resembles that of a specific hapten–antibody system[18,19]. A number of mice myeloma proteins that bind phosphorylcholine share idiotypic determinants with induced anti-phosphorylcholine antibodies indicating the antibody function of these immunoglobulins[18]. Although McPC 603 IgA does not share this idiotypic specificity it binds phosphorylcholine. Crystallographic analysis of the Fab–phosphorylcholine complex revealed that phosphorylcholine binds close to heavy chain residues, occupying a relatively small region of the combining site. No major conformational change was observed in the Fab structure after ligand binding. IgG New binds a number of ligands such as menadione, orceine, uridine and others with a low affinity constant ($K_0 = 1 \times 10^3$ L/M), and a γ-hydroxy derivative of vitamin K_1 (vitamin K_1OH) with a higher affinity constant ($K_0 = 1.7 \times 10^5$ L/M)[20]. Crystallographic studies[21] of the complexes between these ligands and Fab' New showed that vitamin K_1OH binds at the combining site, making contact with a number of amino acid chains of the hypervariable regions of the L and H chains; the other ligands bind at the same site occupied by the naphthoquinone moiety in the vitamin K_1OH–Fab' New complex. Since the affinity constants for these compounds are lower than that observed for vitamin K_1OH it was concluded that the total binding energy in the Fab' New–vitamin K_1OH complex is derived from contacts made by the naphthoquinone moiety and by the phytyl chain with the side chains at the active site. As in the case of the McPC 603 Fab, no major conformational change took place after binding of vitamin K_1OH to Fab' New. These studies provided firm experimental evidence for the concept that the hypervariable regions of H and L chains determine the conformation of the combining site and a preliminary structural model for the interactions between antigens and antibodies. Ligand binding studies have also been conducted with the Mcg L-chain dimer

which has been postulated to represent a primitive antibody molecule[22]. By crystallographic analysis a number of ligands were shown to bind in the vicinity of hypervariable and constant residues of the Mcg L chain.

Using the amino acid sequence and the three-dimensional structure of Fab' New as a basic structural model, a correlation between the structure and function of the well characterised MOPC 315 anti-2,4-dinitrophenyl (DNP) mouse myeloma protein has been made[8]. The L (λ) chains of IgG New and MOPC 315 IgA are highly homologous in their first and third hypervariable regions where they contain a similar number of amino acid residues; also, the sequences of V$_H$ New and V$_H$MOPC 315 are closely related in number of amino acid residues at their hypervariable regions. Therefore, the V$_L$ and V$_H$ sequences of MOPC 315 can be made to fit the basic structure of the active site of IgG New. In this tentative model, MOPC 315 IgA has a shallow combining site with a high density of Trp, Tyr and Phe residues in close correlation with the observed specificity of MOPC 315 IgA for DNP, menadione, flavin mononucleotide, vitamin K_1 and other haptens which include aromatic rings in their structure[23]. IgG New and IgA MOPC 315 have nearly equal avidities for vitamin K_1 and each binds additional ligands not bound by the other. This observation of two binding sites that partially overlap in their ligand-binding spectrum can be taken as support for the postulate that antibodies are multispecific[24,25]. Thus, different antibodies will cover a wide spectrum of antigens with partially overlapping domains of specificity, leading to degenerate immune responses. The mode of binding could be common to a group of chemically related ligands such as DNP, vitamin K_1, menadione, and so on, which share aromatic ring structures. Different affinity constants will be observed in these ligand–antibody complexes reflecting a different number of additional chemical contacts and interactions. A gene pool encoding these multispecific antibodies would be smaller than one encoding monospecific antibodies and each of its structural genes would naturally be subject to repeated induction and expression. The selective advantages of carrying such a genetic pool would ensure its maintenance against genetic drift and spontaneous deleterious mutations.

Three-dimensional structure and variation in immunoglobulin sequences

Variations in the amino acid sequences of the L and H chains can be analysed in terms of their three-dimensional structure. A striking example of the relationship between a deletion (or insertion) and the tertiary structure is observed at positions 27a, 27b and 27c in human L (λ) chains. These residues form part of a single turn helical loop which can be deleted without creating a gap or other major alteration in the path of the polypeptide chain[7]. Deletions also occur in segments of the polypeptide chains of immunoglobulins outside the hypervariable regions. Two examples with a clear structural correlation can be given

here. The first is that of the deletion of residues numbered 54 to 60 in the amino acid sequence of the L chain of IgG New[7]. These residues very nearly constitute the loop of polypeptide chain which is characteristic of the variable regions (V_L, V_{II}) of immunoglobulins and which does not occur in the constant regions C_L, C_H1 (Fig. 1). An integral deletion of this loop can be easily visualised in a three dimensional model of V_L (Fig. 2) and evidently results in a stable tertiary structure similar to that of C_L. A second example is that of the deletion of an entire homology region, which occurs in H chains synthesised by human tumour cells in the "heavy chain disease"[26] and in murine myeloma H chains[27,28], beginning at the start of the C_H1 region (position 119) or before, and ending at position 215, close to the beginning of the C_H2 region. In the three dimensional structure the sequence . . . Val-Ser-Ser shared by γ and μ chains[29], clearly constitutes the end of V_H, and after a sharp bend, position 119 marks the beginning of the C_H1 homology subunit. Complete deletion of C_H1 should not affect the molecular interactions of C_H2 and C_H3, leading to a compact, stable Fc-like molecule with an N-terminal appendix of variable length.

Variations in the amino acid sequences of V_L and V_H which do not affect the length of the polypeptide chains ("point mutations") also have structural correlations[8]. For example, invariable or semi-invariable glycine residues occur at bends (hairpin loops) of the polypeptide chains or at internal points of close contact between two strands of polypeptide chain where larger side chains could not be accommodated; other constant or nearly constant residues are involved in close contacts between V_H and V_L (discussed above), make intrachain hydrogen bonds, or occur in the tightly packed interior of the structure between two β sheets. Multiple mutations that alter these residues to other sequences compatible with an energetically stable, constant three-dimensional structure can best be explained by a process of evolutionary germ-line gene divergence rather than by selection of random somatic mutations. In contrast, the amino acid residues that constitute the hypervariable regions and the antigen complementarity site are not subject to visible structural constraints.

A comparison of the amino acid sequence of β_2-microglobulin with those of the constant regions of immunoglobulins reveals a striking homology[30] indicating that the overall polypeptide chain folding of β_2-microglobulin is similar to the folding of the C regions of immunoglobulins (Fig. 2). This homology includes the hydrophobic amino acid side chains by which C_L and C_H1 interact to form a compact C_1 structural domain. The extent of sequence homologies with the C regions of immunoglobulins further suggests that β_2-microglobulin interacts with another polypeptide chain that shares the basic immunoglobulin fold (Fig. 2). This common structural folding may be reflected in the amino acid sequences of the proteins bearing the murine H-2K, H-2D or the human HLA-K, HLA-D antigenic specificities and which interact with β_2-microglobulin in cell membranes. The structural similarities

between histocompatibility antigens and immunoglobulins suggest a common genetic origin and subsequent steps of translocation and duplication[31].

The basic polypeptide chain folding of immunoglobulins (Fig. 2) has recently been observed in the Cu, Zn enzyme superoxide dismutase from bovine erythrocytes[32]. A comparison of the structure of superoxide dismutase with that of the C and V regions of immunoglobulins indicates that the basic framework pattern of β sheets is present in all three; in superoxide dismutase and in the V regions there are insertions (such as in the hypervariable regions) which presumably have been positively selected through evolutionary events to optimally fulfil a specific biological function. Since the superoxide dismutase activity is present in many organisms including prokaryotes the "immunoglobulin-fold" may indeed turn out to be the expression of a widespread, ancient gene.

1 Krause, R. M., *Adv. Immun*, 12, 1 (1970).
2 Hill, R L., Delaney, R , Fellows, R. E., Jr, and Lebowitz, H. E , *Proc natn. Acad Sci. U.S.A*, 56, 1762 (1966).
3 Kabat, E. A , and Wu, T. T , *Ann N Y Acad. Sci* , 190, 382 (1971)
4 Oudin, J , *J. Cell. Physiol.*, 67, suppl 1, 77 (1966).
5 Kunkel, H. G., Mannick, M , and Williams, R. C , Jr, *Science*, 140, 1218 (1963).
6 Poljak, R. J , Amzel, L. M., Avey, H. P., Becka, L N., and Nisonoff, A , *Nature new Biol*, 235, 137 (1972)
7 Poljak, R. J., *et al.*, *Proc. natn. Acad Sci. U S A.*, 70, 3305 (1973).
8 Poljak, R J , Amzel, L. M., Chen, B L , Phizackerley, R. P , and Saul, F., *Proc. natn. Acad. Sci. U.S.A.*, 71, 3440 (1974).
9 Schiffer, M , Girling, R. L , Ely, K. R , and Edmundson, A B , *Biochemistry*, 12, 4620 (1973)
10 Segal, D M., *et al.*, *Proc. natn. Acad. Sci U S.A.*, 71, 4298 (1974).
11 Epp, O , Colman, P., Fehlhammer, H , Bode, W , Schiffer, M , and Huber, R., *Eur J. Biochem* , 45, 513 (1974)
12 Yguerabide, J , Epstein, H F , and Stryer, L., *J. molec. Biol.*, 51, 573 (1970)
13 Dorrington, K J , and Tanford, C., *Adv Immun* , 12, 333 (1970).
14 Capra, D J , and Kehoe, J. M , *J. Immun.*, 114, 678 (1975)
15 Stevenson, G T , and Mole, L. E , *Biochem J* , 139, 369 (1974).
16 Poljak, R J , Amzel, L. M., Chen, B. L , Phizackerley, R. P , and Saul, F., *Immunogenetics* (in the press).
17 Weigert, M G , Cesari, I. M , Yonkovich, S J., and Cohn, M., *Nature*, 228, 1045 (1970).
18 Potter, M , *Semin. Hematol.*, 10, 19 (1973)
19 Seligman, M., and Brouet, J C , *Semin Hematol* , 10, 163 (1973).
20 Varga, J. M., Lande, S , and Richards, F F., *J. Immun.*, 112, 1565 (1974)
21 Amzel, L M , Poljak, R J , Saul, F , Varga, J., and Richards, F. F , *Proc natn. Acad Sci U.S A* , 71, 1427 (1974).
22 Edmundson, A B., *et al* , *Biochemistry*, 13, 3816 (1974).
23 Michaelides, M. C , and Eisen, H. N , *J. exp. Med* , 140 687 (1974).
24 Richards, F F., Konigsberg, W. H., Rosenstein, R. W., and Varga, J. M., *Science*, 187, 130 (1975)
25 Poljak, R J , Amzel, L. M , Chen, B L., Phizackerley, R P., and Saul, F., *Proc. R Soc* , B (in the press)
26 Wolfenstein-Todel, C , Mihaesco, E., and Frangione, B , *Proc. natn. Acad Sci. U S A.*, 71 974 (1974)
27 Milstein, C , Adetugbo, K , Cowan, W J., and Secher, D S , *Progr Immun.*, (edit by Brent, L , and Holborow, E J), 2, 157 (North Holland, Amsterdam, 1974).
28 Preud'homme, J. L , Birshtein, B. K. and Scharff, M. D , *Proc. natn Acad. Sci U.S A* , 72, 1427 (1975)
29 Florent, G., Lehman, D , and Putnam, F W., *Biochemistry*, 13, 2482 (1974).
30 Peterson, P. A., Cunningham, B. A., Berggard, I , and Edelman, G. M., *Proc. natn Acad Sci. U S A.*, 69, 1697 (1972)
31 Peterson, P. A , Rask, L., Sege, K , Klareskog, L , Anundi, H , and Ostberg, L , *Proc. natn Acad Sci. U.S A* , 72, 1612 (1975).
32 Richardson, J S , Thomas, K. A , Rubin, B. H., and Richardson, D. C., *Proc. natn Acad Sci. U S A* , 72, 1349 (1975)
33 Poljak, R. J , *Contemporary Topics in Molecular Immunology*, 2 (edit by Reisfeld, R. A., and Mandy, W J), 1 (Plenum, New York, 1973)
34 Gally, J A , and Edelman, G. M., *A Rev. Genet.*, 6, 1 (1972)

Signal-to-noise ratio of electron micrographs obtained by cross correlation

J. Frank & L. Al-Ali

Cavendish Laboratory, Cambridge, UK

The signal-to-noise ratio of electron micrographs can be determined by two-dimensional digital cross correlation even though neither signal nor noise can be analysed separately. Such measurements suggest how best to make use of the electron microscope.

HIGH resolution electron micrographs contain a large amount of detail that is not related to the object structure and is commonly referred to as noise. The relative amount of significant information compared with noise in the image can be expressed in terms of the ratio of signal variance to noise variance, or signal-to-noise (s/n) ratio. The s/n ratio is an important criterion for

the quality of the electron micrograph, and for the imaging process by which it is obtained. Unfortunately, no exactly defined electron microscopic model object is available from which the relative portion of the signal variance in the image could be inferred; hitherto, therefore, the s/n ratio has been a merely theoretical concept. Using a cross-correlation technique, we have studied the defocus dependence of the s/n ratio for a carbon film micrograph and found a characteristic behaviour which closely follows the predictions of linear contrast transfer theory, if one takes into account the effects of partial coherence and energy spread and assumes purely elastic scattering.

Our results support the view that, for thin biological objects and bright field illumination, elastic scattering plays the dominant role in contrast formation. They also stress the importance of the underfocus range for practical work and suggest a way of achieving accurate reproducible focusing of instrument with on-line image readout.

The technique

It has been suggested[1] that the sample cross-correlation coefficient could be used for estimating the s/n ratio of band-limited stochastic time functions, if two different versions of the desired signal disturbed by uncorrelated noise are available, each as a series of N time samples taken at a rate equal to twice the bandwidth. The sample cross-correlation coefficient of two series x_i, y_i with zero lag is defined as

$$r_N = \frac{\langle(x_i-\langle x_i\rangle)(y_i-\langle y_i\rangle)\rangle}{\{\langle(x_i-\langle x_i\rangle)^2\rangle\langle(y_i-\langle y_i\rangle)^2\rangle\}^{1/2}}$$

where the angle brackets denote averaging. Assuming stationary Gaussian zero-mean statistics for both signal and noise one obtains the s/n estimate

$$\alpha = \exp[-2/(N-3)](r_N/(1-r_N)+\tfrac{1}{2})-\tfrac{1}{2}$$
$$\approx r_N/(1-r_N) \quad \text{for large } N \qquad (1)$$

The formula for large N can be shown to be identical with the formula that generally relates the s/n ratio of two continuous series to their cross-correlation coefficient, irrespective of the statistical distribution of noise and signal. In image processing application where N is of the order of 10,000, the Gaussian assumption can therefore be dropped.

We have adopted this method of s/n measurement for the quantitative analysis of electron micrographs of a thin amorphous carbon film, and found excellent agreement between the defocus dependence of α obtained from two-dimensional digital correlation, and the theoretical behaviour expected for weakly elastically scattering objects ('weak phase objects').

In these conditions, the action of the electron microscope can be considered[2] as that of a linear system with transfer function $H(k)$ (k spatial frequency), with the projected potential of the object as 'input' and the image intensity recorded on the photographic plate as 'output'. The transfer function[3] describes the distorting and resolution limiting effects due to spherical aberration, defocusing, axial astigmatism, energy spread[4] and partial coherence [5,6]. Assuming an input with power spectrum $P(k)$ and additive signal-independent noise on the output side, the s/n ratio of the output becomes proportional to the signal variance[7].

$$\text{var}(s) = \int |H(k)|^2 P(k)\mathrm{d}k \qquad (2)$$

where the integration extends over the range of spatial frequencies within the bandlimit of the system.

Studies of carbon film

Pairs of electron micrographs of a thin (≈ 100 Å) carbon film were available, each pair taken with a different defocus but in otherwise identical conditions. Assuming a stable specimen, each pair contains the same signal portion distorted by the instrument in a characteristic way, with a superposed noise

portion that is due to the photographic grain and electron noise and is by first approximation additive to the signal. For the experiment, a JEM 100B electron microscope was used at 100 kV, ×200,000 magnification and large objective aperture (≈ 0.5 Å^{-1}). Defocus values and astigmatic focus difference (250 Å) were determined by optical diffraction using Thon's[8] method of evaluation, whereas the divergence of illumination (semiangle 2.7×10^{-4} rad) and the energy spread (1.8 eV) were obtained from bandlimit measurements (J.F., unpublished) using Young-type optical interference[9] for each micrograph pair. From these parameters, the transfer function can be constructed for any defocus value (Fig. 1). As power spectrum, the intensity curve for elastic scattering of thin amorphous carbon films as obtained by electron diffraction[10] was used. The overall behaviour of this function can be approximated in the interesting spatial frequency range by $P(k)\sim\exp(-|k|/\mathscr{H})$ with $\mathscr{H} = 0.18$ Å^{-1}. Since the highest bandlimit observed in the optical interference experiment was 0.38 Å^{-1}, the integration limit for the computation of the integral (2) was set at 0.4 Å^{-1}. For simplicity, the transfer functions were taken to be rotationally symmetric. As the astigmatism is very small, this approximation was expected to cause no appreciable error. The theoretical s/n ratio so obtained is shown in Fig. 2 as a function of defocus. For comparison, the case of a 'white' object spectrum $P(k) =$ constant was also considered (Fig. 3).

Broadly, the s/n ratio increases with increasing defocus and shows an oscillatory behaviour at positive defocus (that is, underfocus), with the maxima lying just above the defocus positions $[(2n+1)C_s\lambda]^{1/2}$; $n = 0, 1, 2...$ (C_s spherical aberration constant, λ electron wavelength) where large transfer intervals appear in the transfer function. For carbon scattering, the absolute maximum is assumed at $n = 0$ ('Scherzer focus'), whereas it appears at higher defocus values for 'white' scattering. Even if one ignores the oscillations, the curve is remarkably asymmetric about $\Delta z = 0$. Computations with different illumination and energy spread parameters show that this behaviour is typical for imaging conditions where the resolution is limited by illumination divergence rather than energy spread.

The electron micrographs were copied from plates (Ilford EM4) on to fine grain film (Ilford N4E.50). This process is linear within 4% in the interesting density range, as checked by calibration, and does not affect the s/n ratio appreciably. The images were scanned using a Joyce-Loebl Scandig densitometer, with a sampling distance of 25 µm, corresponding to optimum sampling[11] of data with a bandlimit at 0.4 Å^{-1}. The digital data were recorded on magnetic tape and processed using the IMPROC image processing software system[12]. The images were aligned using cross correlation[13], and the orientation checked by rotational correlation techniques[14]. For the computation of the cross-correlation coefficients, matrices of size 128×128 were selected from the scanned data. Since a sizeable portion of the full cross-correlation matrix had to be examined in the context of another study, the computation was done by using the Fourier relation, by forming the conjugate product of the respective discrete transforms, inverse transformation, and appropriate normalisation. As a check on the assumption of stationarity, the cross-correlation coefficient was computed for different areas of the scanned image field, and was found
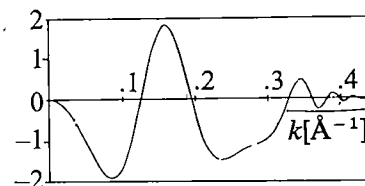
Fig. 1 Electron microscopic phase contrast transfer function for defocus $\Delta z = 1,860$ Å, spherical aberration constant $C_s = 1.8$ mm, electron wavelength 0.037 Å, chromatic aberration constant $C_c = 1.4$ mm, energy spread halfwidth $\Delta E = 1.8$ eV, and Gaussian source distribution with halfwidth 5.4×10^{-4} rad. The transfer function is computed from the coherent transfer function by using the envelope representations [5,6].
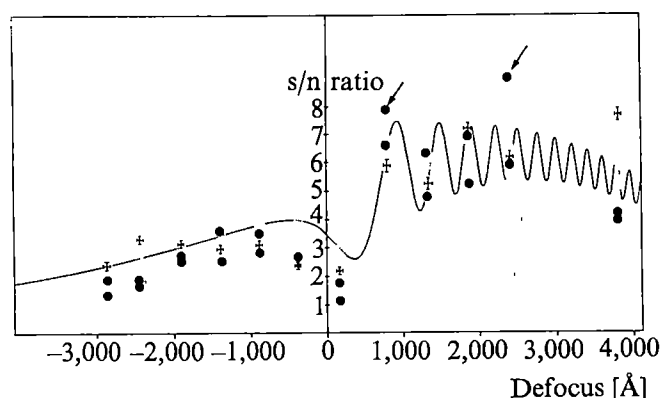
Fig. 2 Theoretical s/n ratio of electron micrographs of carbon as a function of defocus, and experimental s/n ratios α and $\tilde{\alpha}$ of a defocus series obtained from cross correlation (error bars) and from variance measurements (dots), respectively. The theoretical curve was scaled to fit the α measurements at $\Delta z = 790, 1,320, 1,860$ and $2,390$ Å optimally. The sections of the theoretical curve which lie within the horizontal error margins of the α measurements are marked by dotting.

reproducible within 5%. The s/n ratio α was calculated using formula (1). The result was corrected for the decrease of the s/n ratio due to the densitometer. Detailed analysis leads to a correction formula of the form

$$1 + 1/\alpha_{true} = (1 + 1/\alpha)/(1 + 1/\alpha_d)$$

where the s/n ratio of densitometer measurement, α_d, is obtained from the cross correlation coefficient of two scans of the same image. For our measurements, α_d was found to be between 50 and 90. The resulting α_{true} values are plotted in Fig. 2, with the horizontal bars indicating the inaccuracy of defocus determination, estimated at ± 50 Å, and the vertical bars the error limits due to the α_d range and the fluctuation of r when measured in different areas. The scaling factor for the theoretical curve was chosen in such a way that optimum fit occurs for the measurements at $\Delta z = 790, 1,320, 1,860$ and $2,390$ Å.

Interpretation

Theoretically, we expect some deviation of the measured values from the theoretical curve because the latter does not account for the change of noise variance with the signal variance that can be expected for a Poisson process. The defocus dependence of the experimental values is nevertheless in excellent agreement with the predicted behaviour: the measured s/n values are low and follow a flat curve at negative defocus, they then jump to a high value between the Gaussian and the Scherzer focus, and follow the predicted variations at the sampled defocus positions. (Corroborating the detailed structure of the theoretical curve at positive defocus by experimental data would be a formidable task with the method used, involving the evaluation of dozens

Fig. 3 Theoretical s/n ratios of electron micrographs for 'white' object scattering with arbitrary scale.
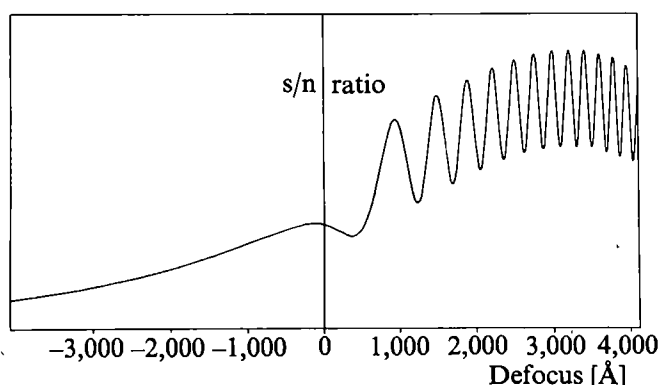
of micrograph pairs. It would be more adequate for this purpose to analyse electron micrographs obtained with a tilted stage which contain the entire defocus range of interest at once[15]. Data of this type are being evaluated at present, so that results on the continuous defocus dependence of the s/n ratio will soon be available (Kübler and Downing, private communication).) As an independent check of the experimental values, we have computed the variances of all image pairs var(i) ,and the variance of an image obtained with free electron exposure in the same optical density range, var(n). From these variances we obtain again with the assumption of additive superposition and uncorrelated noise,

$$\tilde{\alpha} = (var(i) - var(n))/var(n)$$

(Strictly speaking, these s/n estimates should be closer to the theoretical curve than the ones obtained by correlation, since they are obtained with the assumption of signal-independent noise variance.) We found good agreement between α and $\tilde{\alpha}$ (dots in Fig. 2) in the range of small α, and general agreement for large α. The two measurements marked by arrows belong to images whose histograms show a sharp peak on top of a Gaussian appearance. The origin of this artefact, which is missing in the corresponding twin image histograms, is unknown but it is thought that it is responsible for the large deviation of $\tilde{\alpha}$. On the other hand, no reason could be found for deviations at $\Delta z = 1,860$ Å and $3,900$ Å. These discrepancies will be investigated further.

Several conclusions can be drawn from the results of our study: (1) the satisfactory agreement between experimental and theoretical defocus dependence suggests that the linear transfer theory gives an adequate description of contrast formation in the conditions prevailing in the experiment, which are typical for electron microscopy of thin biological specimens; (2) in particular, since the fit of the theoretical curve to the experimental data was achieved by using a scaling factor only, it can be concluded that all those scattering processes ignored in our treatment, which could produce an image with defocus-dependent variance, make only a negligible contribution to the image contrast, such as inelastic scattering[16]; (3) with regard to efforts to retrieve the original object function by restoration techniques, it is important to know that the amount of information is maximised at distinct defocus values. For our experimental conditions, the first four maxima have approximately the same height. On the other hand, it is known from the study of partially coherent transfer functions[17] that with increasing defocus the relative portion of high resolution information passed to the image increases. It may therefore be preferable, for the purpose of subsequent processing, to use defocus values larger than the Scherzer focus in the experiment, even though the electron micrographs may become less intelligible for direct interpretation; (4) as a practical conclusion, our measurements show that for the normal partially coherent illumination used, the gain in s/n ratio lies between two and three if one goes from overfocus to underfocus; (5) in the underfocus range, the positions of maxima and minima are fixed for a given instrument and may therefore be used as absolute focus references, possibly in connection with an objective current wobbler and on-line image readout, complementing methods recently proposed for controlled focusing[18].

The method of measuring the s/n ratio by cross correlation is not restricted to phase contrast electron microscopy, or electron microscopy for that matter; it could be used quite generally, as a performance criterion of electron optical and light optical systems as well as new imaging and recording techniques.

1 Bershad, N. J., and Rockmore, A. J , *IEEE Trans*, IT, **20**, 112-113 (1974).

2 Lenz, F., in *Electron Microcopy in Material Science*, (edit. by Valdre, U.), (Academic, London, 1971).
3 cf. Hanszen, K.-J., *Adv. opt. Elect. Micr.* **4**, 1-84 (1971).
4 Hanszen, K.-J., and Trepte, L., *Optik*, **32**, 519-538 (1971).
5 Hanszen, K.-J., and Trepte, L., *Optik*, **33**, 182-198 (1971).
6 Frank, J., *Optik*, **38**, 519-536 (1973).
7 Goodman, J. W., *Introduction to Fourier Optics*, (McGraw-Hill, New York, 1968).
8 Thon, F., *Z. Naturforsch.*, **21a**, 476-478 (1966).
9 Frank, J., Bussler, P. H., Langer, R., and Hoppe, W., *Ber. Bunsenges. phys. Chem.*, **74**, 1105-1115 (1970).
10 Brünger, W., and Menz, W., *Z. Phys.* **184**, 271-285 (1965).

11 Rosenfeld, A., in *Picture Processing by Computer*, (edit. by Reinholdt, W.), (Academic, London and New York, 1969).
12 Saxton, W. O., *Comp. Graph. Im. Proc.*, **3**, 266-276 (1974).
13 Langer, R., Frank, J., Feltynowski, A., and Hoppe, W., *Ber. Bunsenges. phys. Chem.*, **74**, 1120-1126 (1970).
14 Saxton, W. O., *Eighth Int. Congr. Electron Microscopy Canberra*, **1**, 314-315 (1974).
15 Krakow, W., Downing, K. H., and Siegel, B. M., *Optik*, **40**, 1-13 (1974).
16 Misell, D. L., *J. Phys. D.*, **6**, L1-L5 (1973).
17 Frank, J., *Optik* (in the press).
18 Frank, J., *J. Phys. E.* (in the press).

E. coli membrane lipid alteration affecting T4 capsid morphogenesis

Lee D. Simon, Thomas J. M. McLaughlin, Dale Snover*, Jonathan Ou, Charles Grisham† & Marilyn Loeb

The Institute for Cancer Research, The Fox Chase Center for Cancer and Medical Sciences, Philadelphia, Pennsylvania 19111

An Escherichia coli *mutant,* hd *B3-1, cold sensitive for* T4+ *capsid assembly is described. The* hd *B3-1 character seems to be caused by altered lipid components of the bacterial inner membrane. The genetic locus for the* hd *B3-1 phenotype is designated* fat A *and is closely linked to proline.*

THE *E. coli* B inner (cytoplasmic) membrane has been implicated in the assembly of bacteriophage T4+ heads. Electron micrographs of sections through cells fixed shortly after infection show amorphous lumps of viral head proteins associated with the bacterial inner membrane. These lumps form intermediate membrane-bound structures called τ particles which mature into empty phage heads that move from the inner membrane to the cytoplasm, where they are filled with DNA[1]. Host factors thus seem to be involved in T4+ head morphogenesis, and we, as well as other investigators, have been able to isolate host cell mutants which enable T4+ phage adsorption but not capsid assembly.

Three *E. coli* strains defective for T4+ capsid formation have been described[2–4]; in these host-defective (*hd*) cells, T4+ head production seems to be blocked at an early stage of assembly. Normal T4+ head precursor proteins are present but are not processed to the cleaved forms found in the mature capsid structures, and only lumps of head proteins are seen on the bacterial inner membrane. Until now, no direct evidence has been presented to indicate the nature of the host block. Specific T4 mutants in gene 31 are able to overcome this host defect in phage formation. In T431⁻ infection, the major capsid protein accumulates in 'lumps' on the *E. coli* inner membrane[5], indicating that the product of gene 31 (p31) may affect the association of T4 head proteins with the host cytoplasmic membrane.

The results presented here describe an *E. coli* mutant, *hd* B3-1, that is cold sensitive for T4+ head formation. Evidence is presented to show that the defect in *hd* B3-1 is associated with an altered lipid component of the cytoplasmic membrane.

Physiology of T4+ infection of *E. coli hd* B3–1

E. coli hd B3-1 is an N-methyl-N′-nitro-N-nitrosoguanidine derivative of *E. coli* B. Within 10 min after the addition of T4+ phage at a multiplicity of infection (m.o.i.) of five phage per cell to mid-log phase *hd* B3-1 bacteria at 30 °C, more than

90% of the cells lose their colony forming ability. *In vitro* complementation tests (data not shown) and electron microscopic studies (Fig. 1) indicate that at 27 °C no detectable T4+ capsids are produced in *hd* B3-1, although tails and tail fibres are made in approximately normal amounts. At 27 °C head assembly does not proceed beyond the stage of membrane-bound lumps; bands of detectable T4+ head proteins, however, are visible on SDS polyacrylamide gels (Fig. 2). At 41 °C, the infected cells produce an average of 50–80 phage per cell.
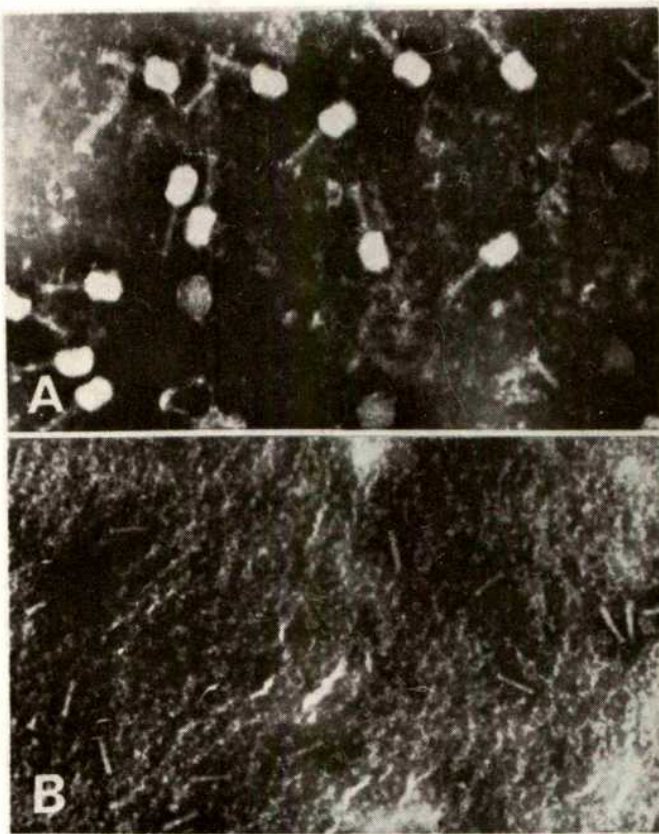
Fig. 1 Electron micrographs of negatively stained crude lysates from T4+-infected *E. coli* B3-1 bacteria. In these and in all subsequent experiments, T4+ refers to T4D phages and unless stated otherwise, mid-log phase bacteria growing in L-broth[1] were used. ×58,500 *a*, Bacteria were grown and infected at 41 °C with T4+ particles at m.o.i. four phages per cell; 5 min later the cells were superinfected with T4+ particles (m.o.i. = 4) to inhibit lysis. *b*, Prepared similarly to *a*, except temperature was 27 °C.

Present addresses: *The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033; †Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901.

a b c d
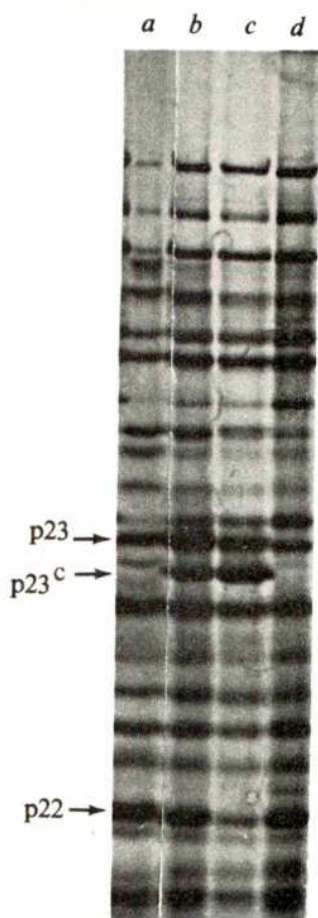
Fig. 2 Autoradiograph of an SDS acrylamide gel showing the phage proteins in T4⁺-infected *hd* B3-1 cells. *E. coli hd* B3-1 and *E. coli* B were grown in LSTG medium (6.4 ml 0.1 M KH₂PO₄, 1.6 ml 0.1 M Na₂SO₄, 2.0 ml 0.5 M MgCl₂, 100 ml 10× low salts, 100 ml 1.0 M Tris, *p*H 7.4, 100 ml 20% glucose, H₂O to 1,000 ml; 10× low salts contains 5.4 g NaCl, 3.0 g KCl, 11.0 g NH₄Cl, 10 ml 0.1 M CaCl₂, 10 ml 0.1 M FeCl₃, H₂O to 1,000 ml). The cultures were then infected (m.o.i. = 3) and super-infected (m.o.i. = 3) 7 min later with T4⁺ particles or with T4*am*H11 (23⁻) mutants. A mixture of ¹⁴C-amino acids (20 μCi ml⁻¹) and ³⁵S-methionine (20 μCi ml⁻¹) was added to the cultures 12 min after infection. The bacteria were pelleted 35 min after infection; the pellets were heated to 100 °C for 2 min in 1% SDS and 1% β-mercaptoethanol. The denatured proteins were run on SDS acrylamide (10%) slab gels for 5.5 h at 80 V, after which the gels were dried. Autoradiographs were then made to enable visualisation of the protein bands. *a* and *b hd* B3-1 infected with T4⁺ phage at 27 and 41 °C, respectively; *c*, B infected with T4⁺ phage. *d*, B infected with T4*am*H11 mutant. p23 and p22 are unprocessed capsid proteins. p23ᶜ is the processed form of p23 found in normal T4 heads; p22 is degraded during capsid morphogenesis²². Following T4⁺ infection of *hd* B3-1 at 41 °C, p23ᶜ is formed and p22 is degraded, whereas at 27 °C, p23ᶜ does not appear and p22 accumulates.

Intermediate temperatures lead to the production of intermediate numbers of complete phage (Fig. 3). Thus, there is a cold-sensitive block in *hd* B3-1 which affects T4⁺ head assembly.

T4⁺ heads can be found soon after shifting a T4⁺-infected *E. coli hd* B3-1 culture from restrictive (30 °C) to permissive (41 °C) temperature 25 min after infection (Fig. 4). Complete phage appear within 4 min after the shift. Conversely, a temperature shift of T4⁺-infected *hd* B3-1 cells from 41 to 30 °C 25 min after infection causes a rapid decrease in phage production (Fig. 4). In other experiments (data not shown) shifting *hd* B3-1 cultures from 20 to 30 °C and from 20 to 41 °C at the time of T4⁺ infection, phage capsids were produced only in the cultures shifted to 41 °C. T4⁺ capsid assembly in *hd* B3-1, therefore, requires temperatures approaching 41° C, regardless of previous culture conditions.

In addition to *E. coli* strains B and *hd* B3-1, the results reported below include *E. coli* B3-1ʳᵉᵛ, a spontaneously arising mutant of *hd* B3-1 which plates T4⁺ phages as efficiently as B between 24 and 41 °C. For all experiments the bacteria were growing rapidly, approximately in mid-log phase (4.5 × 10⁸ cells ml⁻¹).

E. coli hd B3–1 inner membrane

As the host cell membrane seems to play a part in T4⁺ head assembly¹, and as changes in membrane fluidity as a function of temperature may relate to the temperature-dependent *hd* B3-1 block in capsid assembly, we suspected that *hd* B3-1 may possess an altered cytoplasmic membrane. SDS polyacrylamide gels of inner membranes from *hd* B3-1 and B showed identical protein bands. Therefore, we investigated the possible relationship between the host defect and altered inner membrane lipid components.

To determine whether the effect of temperature on T4⁺ capsid production in *hd* B3-1 is related to the mobility of the inner membrane lipids, we used electron spin resonance spectroscopy (ESR). Electron spin-labelled methyl esters of fatty acids

$$I_{m,n} = CH_3(CH_2)_m - \underset{\underset{\displaystyle N \to O}{O}}{C} - (CH_2)_n - COOCH_3$$
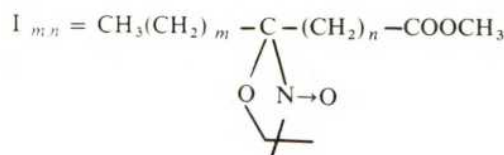
were used to examine the mobility of the lipids of purified⁶ bacterial inner membranes. The membranes were labelled with either 6-(4′,4′-dimethyloxazolidinyl-N-oxyl) heptadecanoate (I₁₀,₄) or 12-(4′,4′-dimethyloxazolidinyl-N-oxyl) stearate (I₅,₁₀); electron spin resonance (ESR) spectra were then
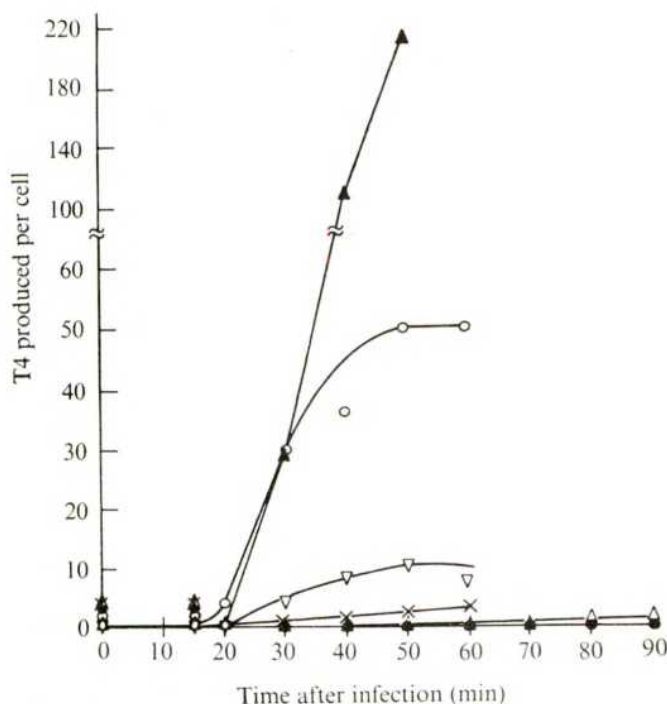
Fig. 3 Phage production following T4⁺ infection of *E. coli hd* B3-1 and *E. coli* B. Bacteria growing at various temperatures were infected with T4⁺ particles (m.o.i. = 5) and 5 min later they were superinfected (m.o.i. = 5). ▲, B 30 °C; ●, B3-1 27 °C; △, 30 °C; ×, 33 °C; ▽, 37 °C; ○, 41 °C. Anti-T4 serum was added 8 min after infection; 10 min after infection the bacteria were diluted × 10⁴ to prevent inactivation of progeny phages by the antiserum. At various times, samples were removed from the infected cultures, exposed to chloroform to lyse the bacteria, and assayed for plaque forming particles to determine phage production.
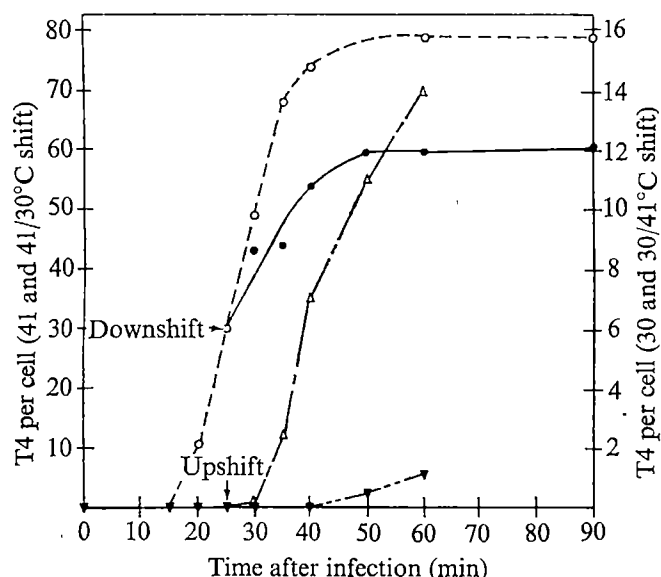
Fig. 4 Effects of temperature shifts 25 min after T4+ infection on phage production in *hd* B3-1. Conditions were similar to those described for Fig. 3, except that 25 min after infection the 30 (▼, △) and 41 °C (○,●) cultures were each divided into two equal parts; one part was maintained at the same temperature whereas the other part was shifted from the initial temperature of 30 to 41 °C (△) or from the initial temperature of 41 to 30 °C (●).

obtained over the temperature range 3–60 °C. The ratio (B/A) of the mid-field peak intensity to the low field peak intensity was taken as a measure of membrane fluidity. B/A values of nearly 1.0 are characteristic of very fluid, isotropic motion of the spin-labelled fatty acid ester, whereas values greater than 1 typically indicate more restricted motion of the spin-labelled molecule.

A discontinuity in a membrane fluidity profile indicates a transition in the physical state of the spin-labelled fatty acid esters. A discontinuity may indicate true phase transition, that is, a melting of the lipid chains where they are in a solid or ordered state below the transition temperature and are in a fluid or disordered state above the transition temperature[7]. Alternatively, a discontinuity may indicate lateral phase separations of the type described by Shimshick and McConnell[8] and Linden et al.[9], which involve the clustering of membrane lipids into solid phases, liquid phases and solid–liquid phase mixtures. Lateral phase separations require the occurrence of rapid lateral motion of lipids in the bilayer; this has been observed in several systems[8,10].

The data obtained with the $I_{10,4}$ label (Fig. 5a) and with the $I_{5,10}$ label (Fig. 5b) indicate that the purified *E. coli* B inner membranes are more fluid than the *hd* B3-1 or B3-1 rev membranes throughout most of the temperature range. Furthermore, discontinuities are observed in all of the fluidity profiles. With the $I_{10,4}$ label, the *E. coli* B and *hd* B3-1 membranes exhibited discontinuities at 38.4 and 48 °C, respectively; with $I_{5,10}$ label discontinuities were seen at 31.7 °C for B and at 47 °C for *hd* B3-1. The B3-1 rev membranes exhibited discontinuities at both 21 and 29 °C with $I_{10,4}$ and at both 19 and 27 °C with $I_{5,10}$. Thus, the temperature at which a discontinuity appears in the membrane fluidity profile is much higher for *hd* B3-1 than for either B or B3-1 rev.

To investigate the chemical bases for these observations, we examined inner membrane phospholipid compositions. Cells were grown for 12 h in medium containing inorganic[32]P-phosphate to label membrane phospholipids. Subsequently, inner membranes were purified from the bacteria; the phospholipids were extracted by the procedure of Bligh and Dyer[11], and the various phospholipid classes were separated by ascending one-dimensional thin-layer chromatography. Phospholipid spots were identified with iodine vapour, ninhydrin, and molybdate reageant[12].

After labelling for 12 h (Table 1) *hd* B3-1 inner membranes had 39% more cardiolipin and 27% less phosphatidylglycerol than B membranes. The B3-1 rev membranes showed only an 11% increase in cardiolipin and only a 19% decrease in phosphatidylglycerol. The other major phospholipid of the *E. coli* inner membrane, phosphatidylethanolamine, showed little if any change in the different membranes.

To characterise further the membrane lipid components, we investigated the fatty acid compositions of the purified inner membranes. As the fatty acids of the *E. coli* inner membrane are contained in phospholipids[13], we prepared free fatty acids from the phospholipids by alkaline hydrolysis. Free fatty acids were methylated with diazomethane and separated by gas–
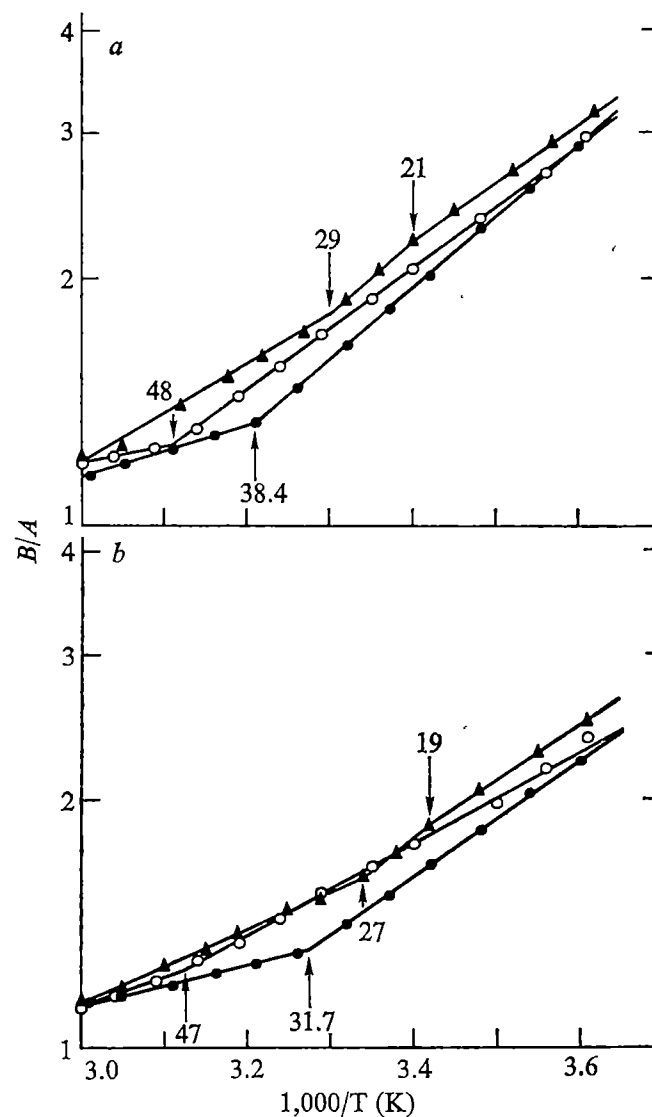
Fig. 5 Temperature dependence of the fluidity (B/A) of spin-labelled fatty acid esters in cytoplasmic membranes from *E. coli* B, *hd* B3-1 and B3-1 rev. Arrows indicate discontinuities in the ESR fluidity profiles. a, Spin-labelled fatty acid ester, methyl 6-(4',4'-dimethyloxazolidinyl-N-oxyl) heptadecanoate $(I_{10,4})$ was prepared by the method of Waggoner et al.[20]. *E. coli* inner membranes, purified by the procedure of Osborn et al.[6] from bacteria growing at 37 °C, were suspended in 3% sucrose, 10 mM Tris, pH 7.5 at a concentration of 10–12 mg membrane protein ml⁻¹. The procedure for incorporation of the spin-labelled fatty acid esters into the purified membranes has been described[18]. ESR spectra were obtained at a frequency of 9,145 MHz on a Varian E4 spectrophotometer equipped with an E4540 variable temperature controller. All samples were 40 μl and were contained in a quartz capillary tube. b, Similar to a, except using methyl 12-(4',4'-dimethyloxazolidinyl-N-oxyl) stearate $(I_{5,10})$ as the spin label. ●, *E. coli* B; ○, *E. coli hd* B3-1; ▲, *E. coli* B3-1.

Table 1 Inner membrane phospholipid composition of *E. coli* B, *hd* B3-1 and B3-1ʳᵉᵛ

	% Total phospholipids after 12 h ³²P label		
	Cardiolipin	Phosphatidylglycerol	Phosphatidylethanolamine
E. coli B	12 5±0.3	18 3±1 1	69 3±0.9
E. coli hd B3-1	17.3±0.1	13.4±0.3	69.3±0.3
E. coli B3-1ʳᵉᵛ	13 8±0.7	14 9±0.5	71 3±0.9

Phospholipid compositions of purified cytoplasmic membranes from *E. coli* B, *hd* B3-1 and B3-1ʳᵉᵛ. Cells were grown overnight at 37 °C in tryptone broth (1.0% Bactotryptone, 0.5% NaCl) with ³²P (1.0 μCi ml⁻¹) after which they were diluted 1:500 and grown in fresh tryptone broth with ³²P (1.0 μCi ml⁻¹) for six generations. Phospholipids were then extracted from purified cytoplasmic membranes[6] by the method of Bligh and Dyer[11]. One-dimensional, ascending chromatography on Baker Silica Gel IB2 sheets (Baker, Phillipsburg, New Jersey) was used for separation of phospholipid classes with a neutral organic solvent system of CHCl₃–CH₃OH–H₂O (65·25:4, v/v). Phospholipid spots were visualised with iodine vapour, ninhydrin (Sigma, Saint Louis), molybdate reageant[12] and autoradiography. Phospholipid spots were cut from the chromatogram and placed in 5 ml Liquifluor (New England Nuclear, Boston) for determination of radioactivity in a Beckmann LS230 scintillation counter.

liquid chromatography on a diethylene glycol succinate column[14]. The proportions of the various fatty acids in our *E. coli* B inner membrane preparations (Table 2) are similar to those reported[15]. The proportions of the various fatty acids extracted from *hd* B3-1 inner membranes, however, are distinctly different from those of B (Table 2). Compared with the membranes of B, those of *hd* B3-1 contained 28% more palmitoleic acid (16:1) plus its cyclopropyl derivative, 9, 10-methylenehexadecanoic acid and 18% less cis-vaccenic acid (18:1) plus its derivative, 11, 12-methyleneoctadecanoic acid. Palmitoleic acid is known to be the direct precursor of cis-vaccenic acid[14]. Therefore, the conversion of palmitoleic acid to vaccenic acid seems to be partially blocked in *E. coli hd* B3-1.

Fatty acids extracted from B3-1ʳᵉᵛ membranes, although containing more 9, 10-methylenehexadecanoic acid, seem to be quantitatively similar to those of B (Table 2). Although there was a 9% increase in palmitoleic acid and methylenehexadecanoic acid in B3-1ʳᵉᵛ, cis-vaccenic acid and its methyleneoctadecanoic acid derivative showed no significant decrease. Comparison of *hd* B3-1 and B3-1ʳᵉᵛ suggests that the altered fatty acid composition of the bacterial inner membrane and the block in phage capsid production are acquired or lost jointly.

Additional evidence supporting the relationship between the *hd* B3-1 host defect and inner membrane lipid alterations comes from the study of certain *E. coli* B transductants. Cotransduction of markers by P1 phages was used to map the *hd* B3-1 gene responsible for the cold sensitive block. The gene, *fat* A, responsible for the *hd* B3-1 character cotransduced with a proline marker at a very high frequency.

We purified the inner membranes from one such transductant (*E. coli* BOU3101) and from the parental strain (*E. coli* B45) and compared their fatty acid compositions. As T4⁺ capsids fail to assemble in the transductant at 27 °C, we conclude from the data in Table 2 that the *E. coli* BOU3101 transductant acquired the inner membrane fatty acid composition as well as the host-defective character of *hd* B3-1. The membranes from transductants that acquired the proline⁺ gene from B3-1ʳᵉᵛ showed normal fatty acid compositions. These studies indicate that both the inability of *hd* B3-1 to produce T4 capsids at low temperatures and the inner membrane fatty acid alterations are controlled by the single genetic locus, *fat* A.

T4⁺ phage infection is known to alter the protein and phospholipid compositions of the *E. coli* membrane[16,17]. To determine whether the differences observed among the inner membranes isolated from uninfected B, *hd* B3-1, and B3-1ʳᵉᵛ were also characteristic of membranes isolated from similar bacteria 15 min after T4 infection, cultures of B, *hd* B3-1 and B3-1ʳᵉᵛ (all of which are *su*⁻) were infected with T4*am*H11 (23⁻) particles. To prevent any differential accumulation of the major capsid protein, p23, on the bacterial inner membranes, T4*am*H11 phage were used instead of T4⁺; 15 min after infection the cells were chilled and converted to spheroplasts[6]; inner membranes were then purified using the procedure for membranes of uninfected cells.

We again extracted free fatty acids from the membrane phospholipids, after which we determined the proportions of the various fatty acids by gas–liquid chromatography. The

Table 2 Inner membrane fatty acid composition (% of total)

Fatty acid	*E. coli* B	*E. coli* B3-1ʳᵉᵛ	*E. coli hd* B3-1	*E. coli* B45	*E. coli* BOU3101
		Uninfected bacteria			
12:0	1.06	0.62	0.38	3.60	1.35
14:0	2.50	2.34	2.94	13.48	7.28
16:0	27.57	29.93	32.00	21.80	32.84
16:1	16.19 ⎱24.34	9.61 ⎱26.48	18.75 ⎱31.16	13 93 ⎱17.84	29.96 ⎱34.62
17C	8.15 ⎰	16 87 ⎰	12.41 ⎰	3.91 ⎰	4 66 ⎰
18:0	2.15	1.85	1.42	3.73	3.03
18:1	34 90 ⎱	30.79 ⎱	28.47 ⎱	32 26 ⎱	19.00 ⎱
UI	(3.38) ⎰38.95	(1.48) ⎰37 32	(1.10) ⎰30.99	— ⎰38 99	— ⎰20 91
19C	4.05 ⎰	6.53 ⎰	2 52 ⎰	6.29 ⎰	1.91 ⎰
UII	—	—	—	—	—
		T4*am*H11-infected bacteria			
Fatty acid	*E. coli*. B	*E. coli* B3-1ʳᵉᵛ	*E. coli hd* B3-1		
12:0	2.05	3 25	1.10		
14:0	2.71	4.41	3 13		
16:0	30.68	25.89	31.32		
16:1	17.54 ⎱24 10	15 20 ⎱20.47	21.30 ⎱30.38		
17C	6 56 ⎰	5 27 ⎰	9.08 ⎰		
18:0	1.88	3.47	3.21		
18:1	33.06 ⎱	29.69 ⎱	26.31 ⎱		
UI	(2.14) ⎰37 52	(1.27) ⎰34.21	(1.57) ⎰27.88		
19C	4.46 ⎰	5.52 ⎰	1.57 ⎰		
UII	—	5 81	1 41		

Fatty acid compositions of *E. coli* B, *hd* B3-1, B3-1ʳᵉᵛ, B45, and BOU3101 inner membrane phospholipids. Inner membranes were purified[6] either from uninfected bacteria (4.5 × 10⁸ ml⁻¹) growing at 37 °C or from cells which had been infected with T4*am*H11 (23⁻) particles (m.o.i. =5). Infected bacteria were centrifuged for 1 min at 12,000*g* 15 min after addition of phages. Membranes were then prepared in the usual way The phospholipids of the purified inner membranes were extracted by the method of Bligh and Dyer[11]. Free fatty acids were prepared from the phospholipids by alkaline hydrolysis[18], followed by immediate methylation with diazomethane The resultant fatty acid methyl esters were separated on a diethylene glycol succinate column in a Hewlett Packard 5700A gas chromatograph equipped with a chart integrator. Column temperature, 175 °C. N2 was used as the carrier. Quantitative mixture KD (Applied Science L., State College, Pennsylvania) was used for fatty acid standards. Proportions of the various fatty acids were determined from the areas under the peaks recorded by the chart integrator

results of this experiment (Table 2) indicate that compared with inner membranes from infected *E. coli* B, the inner membranes from infected *E. coli hd* B3-1 cells have a decreased proportion of *cis*-vaccenic plus methyleneoctadecanoic acids and an increased proportion of palmitoleic plus methylenehexadecanoic acids. Furthermore, the membranes from T4*am*H11-infected B3-1 ^{rev} show nearly the same fatty acid composition as the control. These data are very similar to the data obtained from uninfected bacteria.

Implications

The above data indicate that purified inner membranes from *E. coli hd* B3-1 bacteria have altered fatty acid and phospholipid compositions as well as an unusually high viscosity. Further, studies of transductants for the host-defective character and of spontaneous revertants suggest that both the cold-sensitive block in T4 head production and the inner membrane alterations are controlled by the same gene, designated *fat* A. As T4$^+$ heads assemble on the *E. coli* inner membrane[1], we conclude that the cold sensitivity of T4$^+$ capsid formation in *hd* B3-1 is caused by the altered lipid components of the inner membrane.

Cotransduction by P1 phage, rather than conjugation, was used to map the *fat* A locus as numerous attempts to make *hd* B3-1 male (F$^+$) failed. The parent strain, *E. coli* B, was readily made F$^+$ in control experiments. It seems that, in addition to its effect on T4 morphogenesis, the *hd* B3-1 *fat* A membrane lipid alteration blocks a step in the acquisition or expression of the F episome, both at low (30 °C) and at high (41 °C) temperatures.

The *fat* A locus, responsible for the *hd* B3-1 phenotype, seems to be near proline in the *E. coli* B chromosome. Genes coding for proteins involved in fatty acid uptake and degradation are also closely linked to proline[21]. The genetic determinants of the host-defective phenotypes of two other *E. coli* strains in which T4$^+$ capsid production is blocked[2,3] are located near melibiose, far from proline. Therefore, two bacterial genes separated by nearly one-fifth of the *E. coli* chromosome can affect T4 head formation. Both of these genes, however, seem to affect the same cellular structure because T4 mutants in the same gene—gene 31—can overcome either, and possibly both, bacterial blocks. The product of T4 gene 31 functions during the organisation of T4 head proteins on the bacterial membrane[5]. An interesting hypothesis to account for these observations is that the initiation of T4 head assembly

requires specific protein-binding sites on the *E. coli* inner membrane. Capsid production could be blocked either by altering this membrane protein (genetically linked to melibiose) or by changing membrane lipid components (linked to proline) associated with the protein, so that at low temperatures formation of the capsid protein–membrane protein complex would be sterically or allosterically inhibited. At higher temperatures the increased disorder of the membrane lipids might enable the initiation of capsid assembly. An example of this type of effect of membrane lipids on the interaction of a membrane protein with a cytoplasmic molecule is the cold sensitivity of sheep kidney ATPase activity[18]. Changes in the organisation of the cytoplasmic membrane lipids seem to be responsible for the marked reduction in the rate of ATPase activity which occurs below 20 °C.

1 Simon, L. D., *Proc. natn. Acad Sci. U.S A.*, 69, 907–911 (1972).
2 Takano, T., and Kakefuda, T., *Nature new Biol.*, 239, 34–37 (1972)
3 Coppo, A., Manzi, A., Pulitzer, J. F., and Takahashi, H., *J. molec. Biol*, 76, 61–87 (1973).
4 Georgopolous, C P., Hendrix, R. W, Kaiser, A. D , and Wood, W. B., *Nature new Biol.*, 239, 38–41 (1972).
5 Laemmli, U. K , Beguin, F., and Gujer-Kellenberger, G., *J. molec. Biol.*, 47, 69–85 (1970).
6 Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J., *J. biol. Chem.*, 247, 3962–3972 (1972).
7 Heinz, H , and Sturdervant, J , *J. biol. Chem.*, 247, 6071–6075 (1972).
8 Shimshick, E. J., and McConnell, H. M., *Biochemistry*, 12, 2351–2360 (1973).
9 Linden, C D., Wright, K. L., McConnell, H. M., and Fox, C. F., *Proc. natn. Acad. Sci. U.S.A.*, 70, 2271–2275 (1973).
10 Sackmann, E., Trauble, H., Galla, H. J., and Overath, P., *Biochemistry*, 12, 5360–5369 (1973).
11 Bligh, E. J., and Dyer, W. J., *Can. J. biochem. Physiol.*, 37, 911–917 (1959).
12 Dittmer, J. C., and Lester, R. L., *J. lipid Res.*, 5, 126–127 (1964).
13 Cronan, Jr., J E., and Vagelos, P. R. *Biochim. biophys. Acta*, 265, 25–60 (1972)
14 Ackman, R G., in *Methods in Enzymology*, 14, (edit. by Lowenstein, J. M.), 329–381 (Academic, New York, 1969).
15 Kanemasa, Y., Akamatsu, Y., and Nojima, S., *Biochim. biophys. Acta*, 144, 382–390 (1967).
16 Weintraub, S. B , and Frankel, F. R., *J. molec. Biol.*, 70, 589–615 (1972).
17 Merlie, J. P., *Biochimie*, 56, 717–728 (1974).
18 Grisham, C , and Barnett, R., *Biochemistry*, 12, 2635–2637 (1973)
19 Dittmer, J. C., and Wells, M. A , in *Methods in Enzymology*, 14, (edit by Lowenstein, J. M), 482–530 (Academic, New York, 1969).
20 Waggoner, A., Kingzett, T., Rottschaefer, S., and Griffith, O., *Chem. phys. Lipids*, 3, 245 (1969).
21 Klein, K , Steinberg, R., Fiethen, B., and Overath, P. *Eur. J Biochem.*, 19, 442 (1971).
22 Laemmli, U. K., *Nature*, 227, 680 (1970).

β structures of alternating polypeptides and their possible prebiotic significance

André Brack & Leslie E. Orgel

The Salk Institute for Biological Studies, San Diego, California 92112

A survey of the commonest amino acids formed in prebiotic conditions suggests that the earliest form of genetic coding may have specified polypeptides with a strong tendency to form stable β-sheet structures. Poly(Val-Lys), like other polypeptides in which hydrophobic and hydrophilic residues alternate, tends to form β structures. We show that bilayers with a hydrophobic interior and a hydrophilic exterior may be present in aqueous solution.

MILLER and others have published a large number of papers dealing with the prebiotic synthesis of amino acids[1]. The formation of polypeptides from amino acids in prebiotic conditions has also been reported[1]. Since we know a great deal

about the conformational tendencies of the naturally occurring amino acids[2], it should now be possible to deduce the probable conformations of the polypeptides first formed on the primitive Earth.

The assignment of amino acids to trinucleotides by the genetic code is not random. Woese has emphasised that the position of an amino acid in the codon-table is strongly dependent on its hydrophobic or hydrophilic character[3]. Dickerson has suggested that the codon assignments correlate with the tendency of amino acids to occur in the interior or on the surface of globular proteins[4].

Here we bring together conformational information obtained with synthetic polypeptides, information about the abundance of prebiotic amino acids, and the suggestions of Woese and
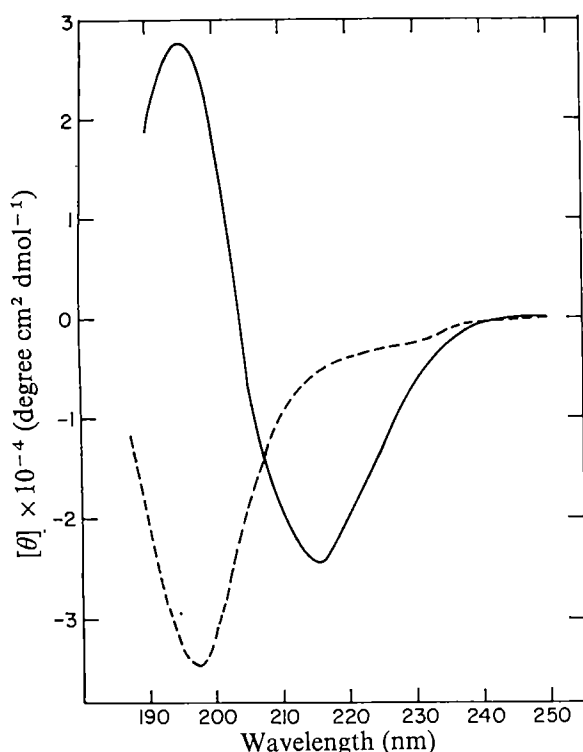
Fig. 1 CD spectra of a freshly prepared solution of poly(Val-Lys) at *p*H 2.3 in water (— — —) and of a solution in 0.1 M NaCl after 60 h (————) at *p*H 2.3.

Dickerson in an attempt to throw light on the origin of the genetic code. In particular, we provide further support for the suggestion that peptides with alternating hydrophilic and hydrophobic residues, that form β-sheet bilayers, may have been important at an early stage in the origins of life[5].

Alternating hydrophobic–hydrophilic residues

A large number of sequential copolypeptides has been synthesised (for a review see Johnson[6]). Among those soluble in water and containing alternating hydrophobic and hydrophilic residues, we are aware of only four that have been submitted to conformational analysis. Three of these polypeptides exhibited the β structure in aqueous solution.

Alternating poly(Glu-Ala) shows a typical β-circular dichroism spectrum after standing for several weeks in neutral aqueous solution[7]. Poly(Tyr-Glu) forms soluble aggregates below *p*H 10.5. Infrared spectroscopy shows that these aggregates have the antiparallel β structure[8]. Seipke *et al.*[9] made a detailed study of the conformation properties of poly(Lys-Phe). At neutral *p*H and low salt concentration the polymer is partly in the β form. The proportion of β form is markedly increased at basic *p*H, or when sodium perchlorate or methanol is added to the solution. The corresponding random copolypeptide poly(Lys, Phe) exhibits a random coil conformation at neutral *p*H, and changes to the β form at higher *p*H, but sodium perchlorate induces a transition to the α helix, as Peggion *et al.* had already shown[10]. The remarkable difference in the behaviour of the two polymers shows that the amino acid sequence can dramatically change the tendency to form a β structure.

Two silks that contain a substantial proportion of alternating residues have been studied in the solid state. It has been shown that the crystalline β region of *Bombyx mori* silk is made up of chains of the type (Gly-X)*n* where X is alanine or occasionally serine. In the structure established by Marsh *et al.*[11] the glycyl residues are located on the same side of the sheets and the packing of successive sheets bring glycyl residues in contact, thus leading to an alternation of glycyl–glycyl and alanyl–alanyl contact layers. Such a structure is also adopted by synthetic poly(Ala-Gly)[12].

Sawflies of the family Argidae produce a silk that contains

roughly equimolecular amounts of alanine and glutamine, accounting for 70–80% of the amino acids. The X-ray diffraction patterns provide evidence that the β structure of this silk is very similar to that found in *Bombyx mori* silk[13].

Conformational analysis of poly(Val-Lys)

Poly(Val-Lys) with a molecular weight of about 5,000 was obtained by polymerisation of the *p*-nitrophenyl ester of the corresponding protected dipeptide. The optical and chemical purity of the sample was confirmed. Details of the synthesis will appear elsewhere.

At acidic *p*H in pure water the CD spectrum of poly(Val-Lys) (Fig. 1) is almost the same as that of random coiled *Bombyx mori* silk fibroin in aqueous solution[14]. We suppose, therefore, that poly(Val-Lys) is a random coil in these conditions. When a 1% or 0.01% acidic solution was brought rapidly to *p*H 8.8 a spectrum typical of the β conformation was obtained. The transition was almost reversible after 15 min at *p*H 8.8. The polymer precipitated from solution above *p*H 9.

When sodium chloride was added to a 1% solution of the polymer at *p*H 2.3 to bring the final NaCl concentration to 0.1 M (the polymer cannot be dissolved in this salt solution) a slow coil → β transition occurred and was almost complete after 60 h (Fig. 1). There was no transition in the absence of added salt, even after one week.

Addition of urea (6 M) to a 1% solution in 0.1 M NaCl at *p*H 4.0 did not prevent the appearance of the β structure, but reduced the rate of the coil → β transition. Guanidine hydrochloride (0.5–6 M) caused the precipitation of the polymer as a β structure at *p*H 4.4

The reduced viscosity of a 1% solution in 0.1 M NaCl at *p*H 2.2 was measured as a function of time. We found a significant decrease of the viscosity with time during the first 5 h (Fig. 2). No change was induced by increasing the *p*H to 7.8 after the solution had equilibrated at *p*H 2.2 for 48 h (Fig. 3). Above *p*H 7.8 the viscosity increased sharply. The appearance of a light turbidity indicated that the polymer begins to precipitate in these conditions.

The meniscus depletion technique was used to determine the apparent molecular weight distribution of the polymer in a 0.05% solution in 0.1 M NaCl at *p*H 5.5, while it was undergoing its coil → β transition. At low speed (12,590 r.p.m.), a first equilibrium was reached which produced molecular weights of about 200,000. At higher speed (59,780 r.p.m.), a molecular weight of 5,200 was measured.

Fig. 2 Time dependence of the reduced viscosity of 1% poly(Val-Lys) in 0.1 M NaCl at *p*H 2.3.
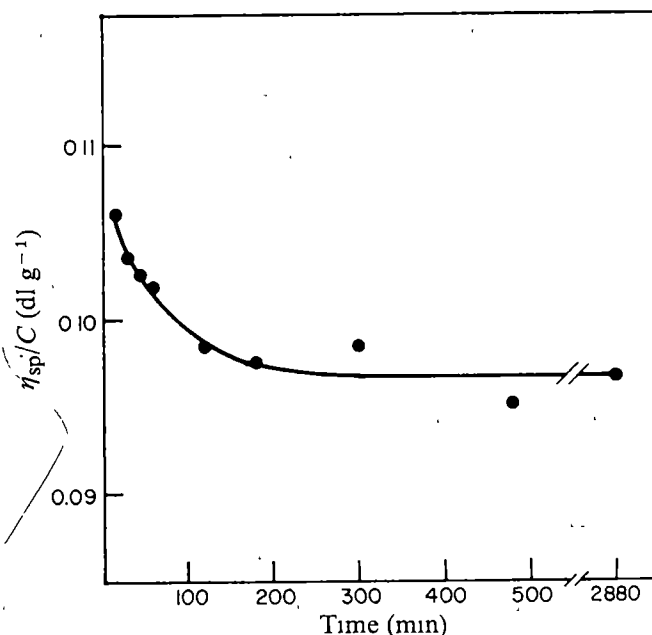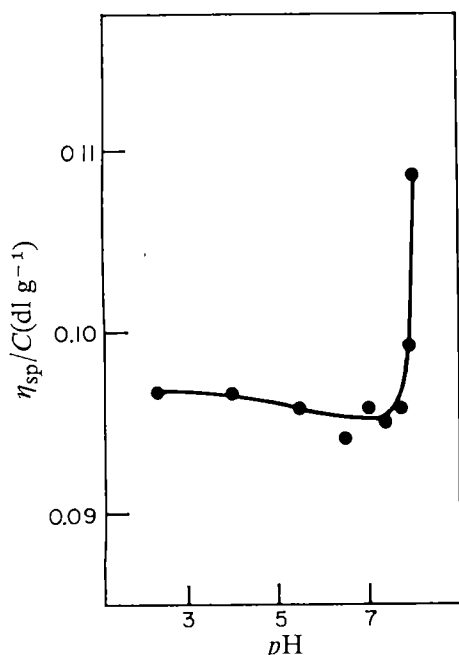
Fig. 3 *p*H dependence of the reduced viscosity of an aged 1% solution of poly(Val-Lys) in 0.1 M NaCl.

The polymer, in the disordered or the β conformation, gave a solution that frothed readily. The surface tension was measured with a Langmuir–Wilhelmy balance. Pure water (72 erg cm^{-2}) and L-α-dipalmitoyl lecithin (0 erg cm^{-2}) were taken as standards. Surface tensions were 30 erg cm^{-2} and 25 erg cm^{-2} for fresh acidic solutions and aged solutions in 0.1 M NaCl at *p*H 3.0, respectively.

Solid state

Dialysis of an aged solution in 0.1 M NaCl at *p*H 2.3 (β form) against 0.5 M sodium perchlorate precipitated the polymer. The product exhibited the characteristic infrared absorption bands of the β structure (amide I band at 1,630 cm^{-1} with a shoulder at 1,690 cm^{-1} indicating an antiparallel arrangement of the chains). The powder pattern of the X rays showed 10 lines, 4 of which were well defined at 26.2±0.5 Å, 8.62±0.6 Å, 4.65±0.03 Å and 3.82±0.02 Å. X-ray studies on the β structure of polyvaline have been reported[15], but without any details. We obtained a powder pattern with strong lines at 9.2 and 4.57 Å, which can be interpreted as the intersheet distance and the interchain distance, respectively.

Implications of conformational studies

The experimental observations described in the previous sections make it clear that the chains of poly(Val-Lys), like those of poly(Lys-Phe), adopt a β conformation in a variety of conditions. We found no evidence for the formation of an α helix, whereas the random coil was stable only in acidic solutions in the absence of added salts.

We do not have any direct experimental evidence as to the spatial organisation of the polypeptide chains into β sheets in solution. We believe, however, that indirect arguments suggest strongly that the bilayer structure shown in Fig. 4c is present rather than sheets of the types shown in Fig. 4a and b.

A striking feature of the X-ray powder photograph of the perchlorate salt of poly(Val-Lys) is the strong reflection at 26.2 Å. Analogy with the diffraction patterns obtained from other β structures[16] shows that this reflection must correspond to the repeat distance perpendicular to the plane of the sheets. The reflection at 4.65 Å corresponds to the usual interchain distance, which commonly lies between 4.62 and 4.78 Å (ref. 16). The reflections at 8.62 and 3.82 Å probably correspond to the 003 and (210+211) reflections, respectively, in an orthorhombic unit cell with *a* = 4.65, *b* = 26.2 and *c* ~6.8 Å.

If we assume this unit cell, it is possible to find a distribution of the atoms in the *ac* plane which predicts a weak 002 reflection, and an intensity ratio for the 001 and 003 reflections close to that observed. The X-ray powder pattern is, however, too poor to enable us to confirm the proposed unit cell.

The phosphate salt of polylysine forms a β-sheet structure with an intersheet distance of 17.16 Å (ref. 17). Our own measurements on the β structure of polyvaline give an intersheet distance of 9.2 Å. If we suppose that the structure illustrated in Fig. 5a would have a repeat equal to the sum of the repeats in the phosphate of polylysine and in polyvaline, we arrive at a value of 26.4 Å, in good agreement with our measured value. On the other hand, the repeat distance for the β-sheet packings illustrated in Fig. 4a and b or for the structure shown in Fig. 5b would be much smaller, probably less than 17 Å. We believe that the X-ray data strongly support a stacked bilayer structure in the solid state and, by inference, a similar bilayer structure in solution.

The behaviour of the polymer in solution is consistent with this interpretation. At *p*H 2.2, in the absence of salt, poly(Val-Lys) exists as a random coil. The addition of small amounts of salt induces a transition to a β structure. This is most simply interpreted as due to the shielding effect of the salt which enables the positively charged amino groups to come together on one side of a β sheet.

The precipitation of the polymer by larger amounts of salt can be explained in a similar way. The formation of a stacked-bilayer structure involves the bringing together of sheets of

Fig. 4 Diagrammatic representation (in chain axis projection) of various arrangement of poly(Val-Lys) chains in β sheets. Ellipses represent the backbone.
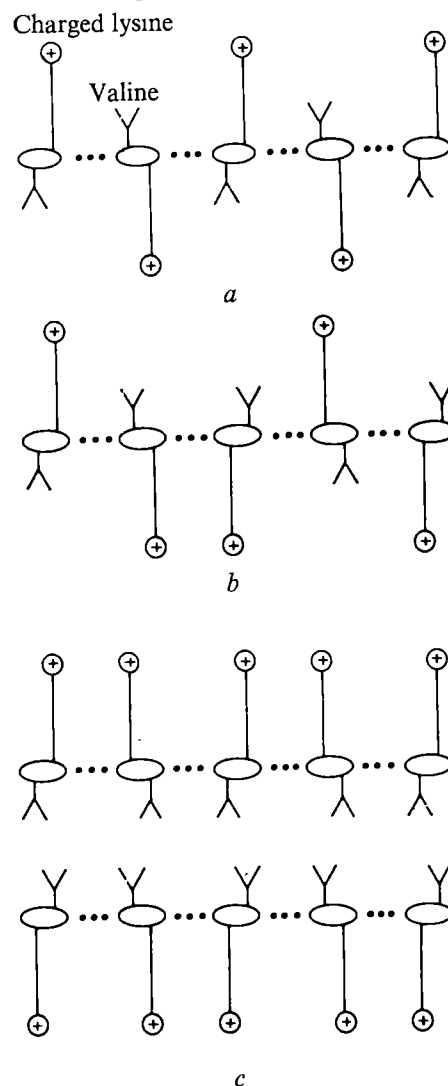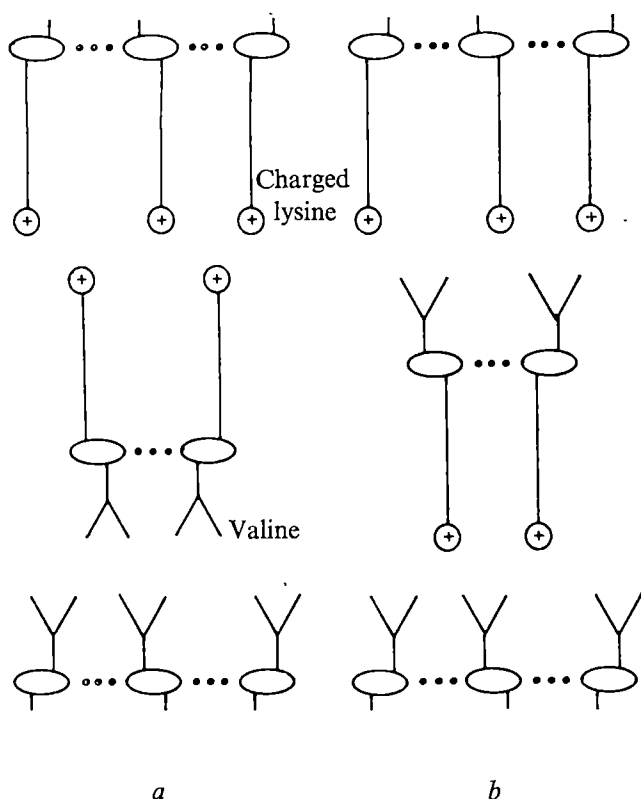
Fig. 5 Diagrammatic representation of two packings of β sheets.

positively charged residues. This is only possible if salt is present in high enough concentration to provide the necessary shielding. The precipitation that occurs in more alkaline solutions (pH 8) is made possible by the neutralisation of the charges on a proportion of the lysine residues.

The high molecular weight (200,000) of the polymer in the β sheet form shows that extensive aggregation has occurred, but throws no light on the spatial arrangement of molecules in the aggregate. Similarly, the drop in viscosity that occurs when the random coil is converted to an equilibrium β sheet in acidic solution gives no detailed structural information.

The β structure which is formed reversibly when freshly-prepared solutions are shifted from acidic to alkaline conditions must be different from the structure formed irreversibly by ageing in acidic solution. Unfortunately, we do not have any evidence as to the detailed structures. We cannot, for example, distinguish between normal β structure and cross β structures.

The surface activity of poly(Val-Lys) is consistent with our hypothesis. At an air–water interface, we should expect the polymer to form a β-sheet monolayer, with the lysine residues penetrating into the aqueous layer and the valine residues exposed to the air. It is also possible, however, that the β structure is disrupted at the interface and that the surface activity is due to the formation of micelles.

The fact that diverse alternating polypeptides form a β structure at all, also provides strong evidence favouring the bilayer structure. It is hard to see what factor other than hydrophobic interaction between residues confined to one side of a β sheet, could account for the general tendency to form β sheets. The formation of a β structure by poly(Lys-Phe), in conditions in which the corresponding random copolymer forms an α helix or a random coil, is particularly striking.

Prebiotic polypeptides

A survey of many experimental studies of the amino acids formed in prebiotic conditions shows that the two simple α-amino acids, glycine and alanine, are formed more readily than any others. In addition, serine, threonine, aspartic acid, glutamic acid and a variety of aliphatic amino acids (including

many that are non-biological) are formed in small and variable amounts. Some α-amino acid derivatives, particularly N-methyl amino acids, and some α-hydroxy and β-amino acids, are also formed. All potentially optically active compounds are obtained as racemic mixture[1].

We do not know if any substantial fractionation of the α-amino acids in the prebiotic mixture took place before condensation of polypeptides occurred. If we suppose that this was not the case, then the first prebiotic condensation products are likely to have been copolymers of glycine and DL-alanine, containing substantial amounts of other α-amino acids. The first peptides might have contained larger amounts of the higher aliphatic amino acids, if enrichment of hydrophobic amino acids was important, or of serine, threonine and the dicarboxylic acids, if hydrophilic amino acids were concentrated.

The conformation of polypeptides containing glycine and alanine have been studied. It is clear that random peptides derived from a mixture of these amino acids would not contain much α helix, if any, since glycine is a strong helix breaker. On the other hand, it seems likely that β-sheet formation would be considerable[18]. The incorporation of N-methyl-α-amino acids and hydroxy acids into the polypeptides would decrease the extent of β-sheet formation, but we are justified in assuming that, in so far as prebiotic 'polypeptides' formed structures at all, rather than random coils, they formed β-sheet structures.

This conclusion is strengthened by the observation that in those cases where a peptide can form either α helices or β sheets, the latter structure is favoured if the molecular weight of the polypeptide is low[19]. Since uninterrupted sequences of α-amino acids in prebiotic condensation products are likely to have been short, β-sheet structures would have had a further advantage over α-helices.

Alternating peptides and the genetic code

The amino acids which are specified by codons with U in the central position—Phe, Leu, Ileu, Met, Val—are all hydrophobic, while those specified by codons with A in the central position—Glu, Asn, Gln, His, Lys, Tyr—are all hydrophilic. The situation is less extreme for the two other groups, but amino acids specified by codons with central C—Ser, Pro, Thr, Ala—tend as a group to be somewhat more hydrophobic than those with a central G—Gly, Ser, Arg, Cys, Trp. Tryptophane and possibly cysteine are exceptions to this rule[3,4].

Studies of mononucleotide–polynucleotide interaction strongly suggest that, in the absence of enzymes, it would be difficult to replicate polynucleotides in which purines are adjacent. Thus polynucleotides in which purines alternated with pyrimidines are likely to have been abundant in the prebiotic soup.

On the basis of these two observations we suggested[5] that the first form of genetic coding 'translated' polynucleotides in which purines and pyrimidines tended to alternate into polypeptides in which hydrophobic and hydrophilic amino acids tended to alternate. Finally, we suggested that alternating polypeptides of this kind were important because they tended to form β sheets with one hydrophobic and one hydrophilic surface. Such sheets would stack together to form 'membrane-like' aggregates. The reader is referred to our previous paper[5] for an amplification of the arguments summarised in this section.

Experimental evidence obtained since our original publication confirms the prediction that alternating polypeptides tend to to form β sheets. Although this by no means proves that our hypothesis concerning the first step in the origin of the genetic code is correct, it suggests that it may be worth looking further for a structural basis for the code. Perhaps the code first assigned glycine to codons with a central purine and L-alanine to codons with a central pyrimidine, while L-serine was associated with both types of codon. This would have generated a crude 'silk'. It may be significant that the amino acids that tend to occur in β turns in globular proteins[2] are coded for by triplets of the form PuPuPy or PyPyPu. We have so far been

unable to provide a convincing explanation of other major features of the genetic code.

1 Miller, S L , and Orgel, L E , in *The Origins of Life on the Earth* (Prentice-Hall, Englewood Cliffs, New Jersey, 1974)
2 Chou, P. Y , and Fasman, G D , *Biochemistry*, 13, 222–245 (1974)
3 Woese, C., in *The Genetic Code* (Harper and Row, New York, 1967)
4 Dickerson, R E , *J molec. Biol.*, 57, 1–15 (1971)
5 Orgel, L. E., *Isr. J Chem* , 10, 287–292 (1972)
6 Johnson, B. J , *J pharm. Sci* , 63, 313–327 (1974).

7 Rippon, W. B., Chen, H H., and Walton, A G , *J molec. Biol.*, 75, 369–375 (1973).
8 Trudelle, Y , *Polymer*, 16, 9–15 (1975)
9 Seipke, G., Arfmann, H. A , and Wagner, K G , *Biopolymers*, 13, 1621–1633 (1974).
10 Peggion, E., Cosani, A., Terbojevich, M , and Borin, G , *Biopolymers*, 11, 633–643 (1972).
11 Marsh, R. E., Corey, R. B , and Pauling, L., *Biochim. biophys. Acta*, 16, 1–34 (1955)
12 Fraser, R D B., MacRae, T. P , Stewart, F H C , and Suzuki, E , *J. molec Biol* , 11, 706–712 (1965)
13 Lucas, F , and Rudall, K. M , in *Comprehensive Biochemistry* (edit by Florkin, M , and Stotz, F H.), 26B, ch. 7 (Elsevier, Amsterdam, 1968)
14 Iizuka, E., and Yang, J. T , *Proc. natn Acad. Sci U S.A* , 55, 1175–1182 (1966)
15 Fraser, R. D B , Harrap, B. S , MacRae, T. P., Stewart, F. H. C , and Suzuki, E , *J. molec Biol.*, 12, 482–490 (1965).
16 Fraser, R D B , and MacRae, T P. in *Conformation in Fibrous Proteins* (edit by Horecker, B , Kaplan, N. O., Marmur, J , and Scheraga, H A.), ch 10 (Academic, New York, 1973)
17 Padden, F. J., Jr, Keith, H D., and Giannoni, G., *Biopolymers*, 7, 793–804 (1969)
18 Astbury, W. T., Dalgliesh. C E., Darmon, S. E , and Sutherland, G B B M., *Nature*, 162, 596–600 (1948)
19 Ananthanarayanan, V. S , *J scient. ind. Res* , 31, 593–612 (1972)

letters to nature

Energy spectrum of hadrons in cosmic rays at sea level

BARUCH *et al.*[1] have measured the energy spectrum of hadrons (nucleons and pions) in cosmic rays at sea level and have presented evidence for an anomalous behaviour in the energy range 2–8 TeV. For $E < 2$ TeV they find the differential vertical intensity to decrease with increasing energy as $E^{-2.6\pm0.1}$, for $2 < E < 8$ TeV the intensity is almost constant, and for $E > 8$ TeV the intensity again decreases with increasing energy (Fig. 1). The 'step' in the spectrum for $2 < E < 8$ TeV is unexpected and Baruch *et al.* propose that it is produced by the interaction of a new particle, of rest mass 40–70 GeV c^{-2}, mean lifetime $> 2 \times 10^{-7}$s and an interaction length of $1,000^{+1,000}_{-700}$ g cm^{-2}. We have made new measurements on the hadron spectrum at the higher energies in question.

The experimental arrangement used consisted of, from top to bottom; a layer of 15 cm of lead; a plastic scintillator (area 1 m²); eight layers of neon flash tubes; 15 cm of iron; a further plastic scintillator (area 1 m²) and 116 layers of neon flash tubes. Hadrons interacting in the lead and iron absorbers produce pions and on the average one-third of these are neutral. Each neutral pion decays into two γ rays and the ensuing electron photon cascade emerges from the absorber and traverses the scintillators. Detailed calculations have been made of the relationship between the average number of burst particles below the absorber and the energy of the initiating particle. The result can be represented approximately by the statement that the mean energy of the 'primary' which produces N particles is N GeV—this relationship is valid over most of the energy range in question; the difference in burst size for incident protons and pions is not large, the size being about 30% larger for pions than for nucleons in iron and 20% larger in lead. The observation of large voltage pulses from either scintillator was used as a master trigger and the pulse heights were displayed on an oscilloscope trace after being delayed by 0 3 μs and 0.9 μs. Subsequently a high voltage pulse was applied to the flash tubes and a photograph showing the geometry of the burst was obtained. Data were taken using different trigger levels and also two different time delays (20 μs and 330 μs) between the occurrence of the burst and the application of the high voltage pulse to the flash tubes. The longer time delay was used for the large burst sizes ($N > 400$ particles), so that it was possible to define the axis of the burst

more precisely—the point being that with such large bursts the total number of particles is very large, as is their lateral spread, and only by reducing the flash tube efficiency can the axis be determined accurately.

All the flash tube layers contained parallel tubes and in the front view bursts were only accepted if their axes passed through a well defined geometrical region and made an angle of $\pm$ 30° to the vertical. The maximum angle a burst could make with the vertical in the back plane was 81° and 75° for the lead and iron targets respectively. Using the method of Lovati *et al.*[2] the measured projected angular distribution of bursts was used to determine the spatial angular distribution, assuming it to be of the form $I(\theta) = I(0) \cos^n\theta$. In this way both n and the vertical intensity $I(0)$ of the burst spectrum were determined. For bursts of size above 400 particles n was found to be 8.0 ± 1.0, in agreement with the expected value.

Conversion from burst size to mean hadron energy was made assuming that all the particles are nucleons; in fact some are pions for which the conversion factor will be slightly different as mentioned already but the ratio of pions to nucleons is not expected to be a rapidly varying function of energy so that the important spectral shape should not be distorted. High energy muons also produce bursts but the effect is small. For a burst size corresponding to 5 TeV hadrons (the centre of the Baruch *et al.* step) the effect is 5% for the lead and $< 1\%$ for the iron.

The final hadron spectrum has been found by averaging the burst spectra from the lead and iron targets (the individual spectra were compatible, adding confidence to our conclusions) and the resulting energy spectrum is shown in Fig. 1 where the hadron spectrum from the measurements of Baruch *et al.*, is also plotted. The rates shown in the figure are absolute rates and no normalisation has been made to other work. Our results show no evidence for a step in the energy range 2–8 TeV and they are in fact consistent with a spectrum of the form $E^{-2.7\pm0.1}$ over the whole energy range from 10 GeV to 10 TeV. There could indeed be an irregularity in the form of the spectrum which would escape detection in this experiment because of the smoothing introduced by the method, by the fluctuations in burst size. To assess the effect of fluctuations we have calculated the probability that a primary proton produces N burst particles per unit N. Assuming the hadron spectrum to be of the form given by Baruch *et al.*, we then calculated the shape of the expected burst spectrum. The result was that the step was clearly present in the burst spectrum. We are therefore convinced that we would have detected the
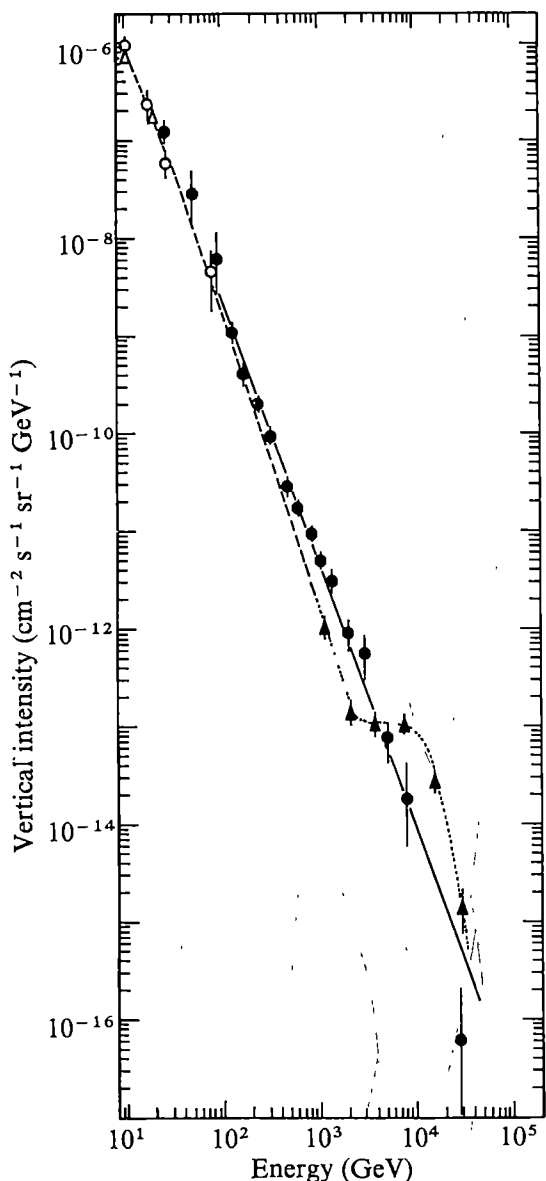
Fig. 1 The differential energy spectrum of hadrons (and of nucleons alone) in cosmic rays at sea level. The low energy data ($\geq$ 100 GeV) are estimates of the total nucleon spectrum found by multiplying the measured proton intensities of Brooke and Wolfendale[3] and Diggory et al[4] by a factor 2 (assuming equal numbers of protons and neutrons). The dashed line is an estimate of the total nucleon spectrum found by increasing the measured neutron intensities summarised by Ashton[5] by a factor of 2. The solid line is the best fit to the present measurement of the energy spectrum of all hadrons. The difference between the energy spectrum of all hadrons (solid line) and the energy spectrum of all nucleons (dashed line) is due to the increasing contribution of charged pions to the total hadron flux for energies > 100 GeV. ▲, Hadrons[1]; ●, hadrons (our work); ○, nucleons[3]; △, nucleons[4].

bump if it was a real effect.

We thank Professor A. W. Wolfendale for encouragement.

F. ASHTON
A. J. SALEH

Department of Physics,
Durham University, Durham, UK

[1] Baruch, J E. F, Brooke, G., and Kellerman, E. W., *Nature*, 242, 6–7 (1973), *Proc. Int Conf Cosmic Rays (Denver)*, 3, 2301–2304 (1973).
[2] Lovati, A., Mura, A , Succi, C , and Taghaferri, G , *Nuovo Cim* ,12, 526–538 (1954)
[3] Brooke, G , and Wolfendale, A. W., *Proc phys Soc.*, 83, 843–851 (1964)
[4] Diggory, J. S , Hook, J. R , Jenkins, I A., and Turver, K E , *J. Phys A*, 7, 741–764 (1974).
[5] Ashton, F., in *Cosmic Rays at Ground Level* (edit. by Wolfendale, A. W),57 (Institute of Physics, London, 1973).

Thermal model of oceanic lithosphere

THE thermal model for oceanic lithosphere developed by McKenzie[1] assumes that oceanic plates are slabs of constant thickness. The model has been used successfully to reproduce both the heat flow and the topographic patterns observed in oceanic lithosphere[2]. There is, however, no reason to assume that the lithosphere has a constant thickness; indeed, it is likely to thicken as it moves away from the ridge and cools.

Thermal models have been derived[3,4] which predict that the thickness, L, of a section of lithosphere should be proportional to the square root of the time, t, since its formation at the ridge, and experiments using spreading layers of wax have confirmed this relationship[5]. Such a model also agrees with the available seismic data (D. Chapman and H. Pollack, personal communication).

Theoretical considerations, using the best estimates for material parameters, predict, however, that the lithosphere should thicken much faster than the available evidence suggests it actually does. This theoretical rate of thickening can be reduced if the effects of the latent heat of the asthenosphere's partial melt are taken into consideration, but to achieve thicknesses equal to those actually observed the asthenosphere must be assumed to be completely liquid[3]. We show here that realistic results can also be obtained if it is assumed that there is a heat flux into the base of the oceanic lithosphere from the underlying asthenosphere.

We have obtained an approximate solution for the temperature field and the thickness of the lithosphere by modifying McKenzie's model. If the lithosphere is viewed in two-dimensional cross section (Fig. 1) it rests on the low-velocity zone, which is taken to be a nearly isothermal zone of partially molten material at a temperature T_m. Except for thin surface layers, the lithosphere is identical to the material beneath it but colder and, therefore, more rigid. The base of the lithosphere is taken as the T_m isotherm. The material beneath the lithosphere is assumed to be capable of maintaining a uniform heat flux, Q, to the base of the lithosphere by convection. The upper surface of the lithosphere is maintained at 0 °C by sea water. At the ridge crest, hot, mantle material accretes to the plate margin. As this material moves away from the ridge it is cooled by sea water. The slab conducts heat away from the mantle beneath it, causing material to freeze on to the bottom of the lithosphere. At equilibrium, the lithosphere attains the proper thickness such that it loses heat upwards as fast as it receives heat from the mantle beneath.

As the lithosphere is very long compared with its thickness and because the temperature difference between its top and

Fig. 1 Predicted shape of the lithosphere plotted in dimensionless variables (see text).
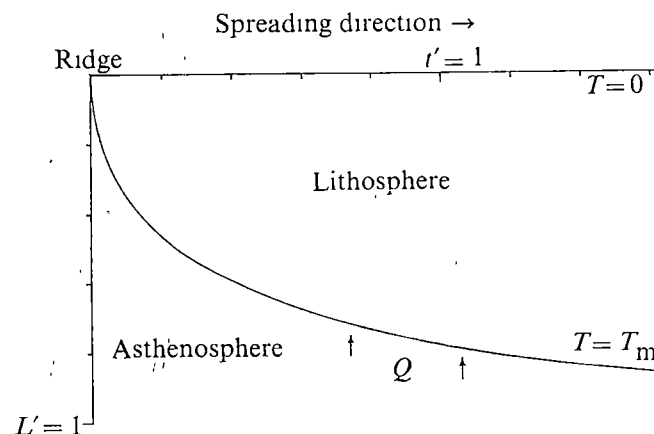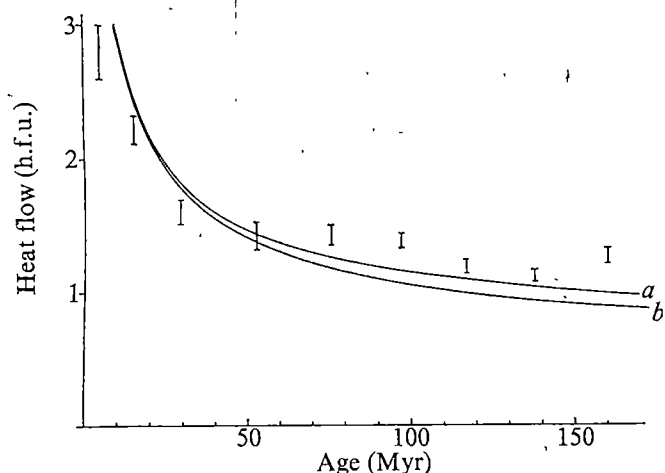
Fig. 2 Observed variation (I) of surface heat flow with age for the northern Pacific[2] compared with theoretical predictions (—). (a, $Q = 0.8$; b, $Q = 0.56$). The error bars indicate the estimated error of the calculated mean and are much smaller than the true scatter of observed values.

bottom surfaces is large, the effects of horizontal heat conduction can be ignored, and the thermal history of a single column of lithosphere as it cools in time can be considered. Assuming that material properties are constant and that there are no radioactive heat sources, the governing heat flow equation is

$$\rho C_p \partial T/\partial t = k \partial^2 T/\partial z^2$$

where T is temperature, z is depth, k is thermal conductivity, ρ is density, and C_p is heat capacity. The boundary conditions are that $T = 0$ at $z = 0$, $T = T_m$ at $z = L(t)$, and $k\partial T/\partial z = Q$ at $z = L(t)$. An approximate solution to this equation can be obtained using a technique developed to handle boundary layers in fluid mechanics. It is assumed that the temperature at any time within a column of lithosphere can be represented by a third order polynomial in z.

$$T(z,t) = a + (bz/L) + (cz^2/L^2) + (dz^3/L^3)$$

where a, b, c, d, and L are functions of time. Considering the boundary conditions together with the fact that $\partial T/\partial t = 0$ at $z = 0$ then $\partial^2 T/\partial z^2 = 0$ at $z = 0$ means that the coefficients can be determined

$$T(z,t) = (3T_m z/2L) - (Qz/2k) - (T_m z^3/2L^3) + (Qz^3/2kL^2) \quad (1)$$

For $Q = 0$ this function is a good approximation to the error function which is the exact solution for this case. At equilibrium the temperature distribution in the lithosphere will be linear in z such that $kT_m/L = Q$, so L will equal kT_m/Q. Substituting this value of L into equation (1) the approximation of the temperature distribution becomes linear as L approaches its equilibrium value.

From equation (1) the heat flow out of the top of the lithosphere at any time is found to be

$$k\partial T/\partial z \,|_{z=0} = (3kT_m/2L) - (Q/2) \quad (2)$$

Neglecting radioactivity, this heat must come from either a deeper source or from the cooling of the slab. At any time, the heat content of a column of lithosphere is

$$\rho C_p \int_0^L T(z)dz = \rho C_p[(3T_m z^2/4L) - (Qz^2/4k) - (T_m z^4/8L^3) + (Qz^4/8kL^2)]\,|_0^L \quad (3)$$

If the slab increases its thickness by ΔL, the original thickness L would have a different, smaller heat content, given by equation (3), with $L+\Delta L$ substituted for L in the denominators of the fractions. The difference between the two values, which is the heat liberated by increasing the thickness of the lithosphere, is $\rho C_p[(3T_m/8) + (QL/4k)]\Delta L$.

In the time interval Δt the heat budget of an individual column of lithosphere is balanced if

$$[(3kT_m/2L) - (Q/2)]\Delta t = \rho C_p[(3T_m/8) + (QL/4k)]\Delta L + Q\Delta t$$

or, in the limit

$$dL/dt = (4/\rho C_p)[(kT_m/L) - Q]/[T_m + (2QL/3k)] \quad (4)$$

The latent heat of the melt solidified to the base of the slab could be included in this balance without difficulty. It is omitted here because if the fraction of partial melt in the asthenosphere is small then its latent heat has a small effect compared with those of the other terms in the equation.

If $Q = 0$, equation (4) has the solution

$$L = (8kt/\rho C_p)^{1/2} \quad (5)$$

which is of the form of previous thickening models. This is also the relationship observed in wax experiments in which there is no deep source of heat.

With Q arbitrary, equation (4) has the solution

$$(5/3)(\ln[1/(1-L')] - L') - L'^2/3 = t' \quad (6)$$

where L' and t' are dimensionless variables

$$L' = LQ/kT_m \qquad t' = t4Q^2/k\rho C_p T_m^2$$

and an arbitrary constant has been fixed so that $L = 0$ at $t = 0$. This solution is plotted in Fig. 1. For small values of
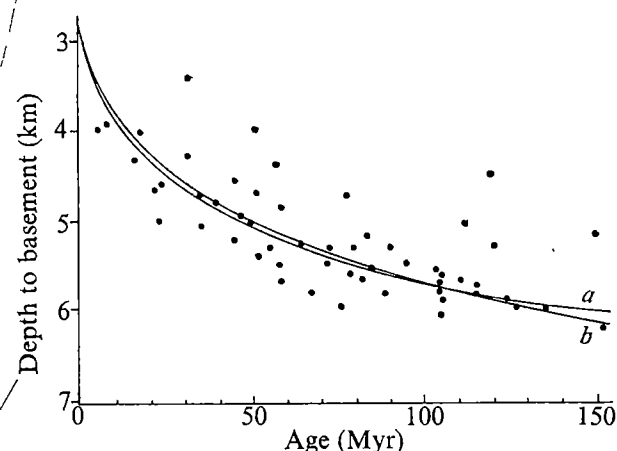
Fig. 3 Observed variation the depth of ocean basement with age[9] compared with theoretical predictions. Data are from all available oceans. ●, Depth determined by drilling; —, prediction (a, $Q = 0.8$; b, $Q = 0.56$).

L', when the contribution from Q is minor, equation (6) reduces to equation (5) as would be expected. As $t' \to \infty$, $L' \to 1$, so $L \to kT_m/Q$ as wanted.

Two numerical examples can be calculated using generally accepted values for material parameters: $T_m = 1,200\,°C$; $C_p = 0.25$ calorie $g^{-1}\,°C^{-1}$; $\rho = 3.3$ g cm^{-3}; $k = 7 \times 10^{-3}$ calorie cm^{-1} s$^{-1}\,°C^{-1}$. For estimates of Q, two values obtained from continental heat flow studies are used: $Q = 0.8$ h.f.u. (0.8×10^{-6} calorie cm^{-2} s^{-1}) represents a probable upper limit to the actual sub-lithospheric flux[6]; $Q = 0.6$ h.f.u. has been suggested as a value compatible with estimates of the heat production of the lower crust and upper mantle[2]. To represent this latter value we use $Q = 0.56$ h.f.u. because it leads to
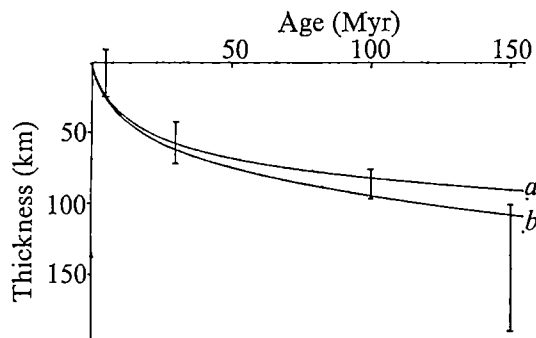
Fig. 4 Com]arison of lithospheric thickness (derived from surface wave studies in the northern Pacific)[10] with theoretical predictions. I.. Seismic estimate; —, prediction (*a*, $Q = 0.8$; *b*, $Q = 0.56$).

convenient sc le factors. With Q equal to 0.8 h.f.u., $L = L' \times 105$ km and $t = t' \times 100$ Myr. For Q equal to 0.56 h.f.u., $L = L' \times 150$ km and $t = t' \times 200$ Myr. The theoretical heat flow curves which result from these material values give a good fit to the data (Fig. 2). Both curves are lower than the data from the older age range, but the difference is only 10–20%. The theoretical curves can probably be raised by that amount if latent heat and radioactivity effects are included in the model.

A theoretical subsidence curve can be calculated by assuming that isostatic compensation takes place at the base of the lithosphere[7]. The mass of any column of water and lithosphere at any time must then equal the mass of the same height of water plus asthenosphere at the ridge. So

$$s(t) = L(t)(\rho_L - \rho_A)/(\rho_A - \rho_w)$$

where $s(t)$ is the subsidence of the surface of the lithosphere below the height of the ridge crest, ρ_w and ρ_A are the densities of seawater and asthenosphere, respectively, and ρ_L is the mean density of the lithosphere. For the case $Q = 0$, ρ_L is constant so $s(t)$ is proportional to $L(t)$. For $Q \neq 0$, the mean density of the lithosphere can be calculated from equation (1) using the assumption that density change results entirely from thermal contraction[7]. The factor $(\rho_L - \rho_A)/(\rho_A - \rho_w)$ becomes $\rho_0 \alpha T_m[(3/8) + (L'(t)/8)]/(\rho_0 - \rho_w)$ where α is the coefficient of thermal volume expansion and ρ_0 is the density at 0 °C. To calculate subsidence curves ρ_0 is taken to be 3.3 g cm^{-3} and α is adjusted so that the subsidence equals 3 km at 100 Myr (Fig. 3). The fit to the available data is quite good. For $Q = 0.56$ h.f.u., α 'must equal 4.0×10^{-5} °C^{-1}, for $Q = 0.8$ h.f.u. it must equal 4.5×10^{-5} °C^{-1}. The measured expansion coefficient for olivine at high temperatures[8] (400–800 °C) varies from $3.1–4.4 \times 10^{-5}$ °C^{-1}, in agreement with the calculated results.

Seismic surface-wave determinations of the depth to the low-velocity zone as a function of the age of oceanic crust[10] are in reasonable agreement with the theoretical predictions (Fig. 4). The error bars in Fig. 4 do not indicate the total uncertainty, but only the allowable variation within the constraints of a preferred model of velocity structure. It is possible that the large thickness calculated to have been obtained after 150 Myr is not real.

As well as providing a physically realistic way of explaining existing heat flow, topographic, and seismic data, this thermal model offers a possible resolution of the oceanic–continental heat flow problem. The surface heat flux in the older oceanic areas is approximately the same as the surface heat flux in the older continental shield areas. Previously, it has been assumed that the older oceanic areas are in thermal equilibrium[2], an idea which was incompatible with the observed near equality of heat flux. As the oceans have much less surface radioactivity than the continents, then if they have the same flux as the

continents at depth they should have a much lower flux at the surface. Our results, however, indicate that the older oceans are not in thermal equilibrium. With a sub-lithospheric heat flux of 0.8 h.f.u., the surface flux at 100 Myr is predicted to be 45% higher than the equilibrium flux. With a sub-lithospheric flux of 0.56 h.f.u., the surface flux at 100 Myr is 85% higher than at equilibrium. Thus, the approximate similarity of surface heat flux in ocean basins and continental shields may be purely coincidental.

I thank Henry Pollack and Dave Chapman of the University of Michigan for the idea to work on this model.

S. THOMAS CROUGH

Department of Geophysics,
Stanford University,
Stanford,
California 94305

1 McKenzie, D. P., *J. geophys. Res.*, 72, 6261–6273 (1967).
2 Sclater, J. G., and Francheteau, J., *Geophys. J. R. astr. Soc.*, 20, 509–542 (1970).
3 Parker, R. L., and Oldenburg, D. W., *Nature phys. Sci.*, 242, 137–139 (1973).
4 Turcotte, D. L., and Oxburgh, E. R., *J. geophys. Res.*, 74, 1458–1474 (1969).
5 Oldenburg, D. W., and Brune, J N., *J. Geophys. Res.* (in the press).
6 Roy, R. F., Blackwell, D. D., and Birch, F., *Earth planet. Sci. Lett.*, 5, 1–12 (1968).
7 McKenzie, D. P., and Sclater, J. G., *Bull. volcan.*, 33, 101–117 (1969).
8 Skinner, B. J., in *Handbook of Physical Constants* (edit. by Clark, S. P.), 92 (*Mem. geol. Soc. Am.*, 97, 1966).
9 Van Andel, T. H., *Earth planet. Sci. Lett.* (in the press).
10 Leeds, A. R., Knopoff, L., and Kausel, E. G., *Science*, 186, 141–143 (1974).

Seawater near the head of the Ross Ice Shelf

THE Ross Ice Shelf (Fig. 1) is a sheet of ice that floats over 540,000 km^2 of the Ross Embayment[1]. The ice in the southern part is between 300 and 800 m thick, and floats on as much as 400 m of water[2,3]. Until now any knowledge of the water mass beneath the shelf has been inferred from studying the open water of the Ross Sea far to the north. Here, I describe a newly discovered natural access to seawater beneath the Ross Ice Shelf, 550 km south of the shelf front and 770 km from the South Pole, on the north-eastern margin of an ice rise.

The ice rise is an elongate north-westerly-trending dome about 40 km long which reaches an elevation of 100 m. The ice

Fig. 1. Location of the rift containing seawater (*a*) near the head of the Ross Ice Shelf, Antarctica. A–A', Section line for Fig. 4; *b*, graben; *c*, crevasse pattern; *d*, sledging route; *e*, ice cliffs 15–20 m high.
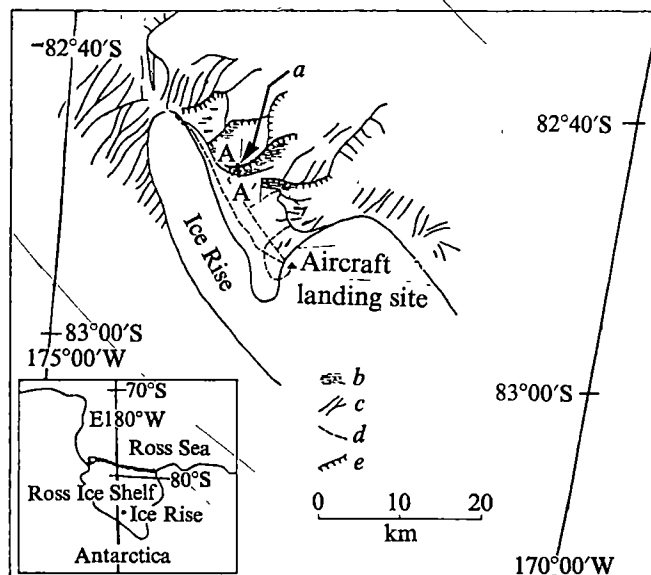
Table 1 Salinity* and freezing point† determinations and cation concentrations‡ for seawater samples from rift shown in Fig. 1

	Conductivity ratio	Salinity (%)	Freezing point (°C)	Cation concentration (‰)			
				Na	Mg	Ca	K
Sample *A*							
Subsample *a*	1.0002	35.01					
b	0.9980	34.92					
c			−1.92				
			−1.98				
Sample *B*							
Subsample *a*	0.9945	34.78					
b	0.9931	34.73					
c				9.63	1.12	0.31	0.45

*Oceanographic Institute, Department of Scientific and Industrial Research, New Zealand.
†Dr A. DeVries, Biology Laboratory, McMurdo Station, Antarctica.
‡Mr P. Kyle, Victoria University of Wellington, New Zealand. Values determined by atomic spectroscopy (error, ± 10%).

of the rise is grounded[2] and the northern and eastern margins are marked by extensive crevassing that continues for many kilometres north of the rise. This crevassing is believed to reflect a flow regime of shearing and extension as the floating shelf moves past the rise on its journey north. Along the north-eastern margin of the rise the ice shelf has broken into plateau-like blocks of ice several kilometres across, separated by broad valleys floored with snowed-in, broken ice ('horst' and 'graben' structures). The southern margin of each block is a continuous

Table 2 Deuterium concentrations of water in the rift

Sample	δ_{SMOW}*	Sample description†
CRL–5	−18‰	Surface water from near Station 1 (Fig. 2)
CRL–6	−11‰	Surface water from near Station 4 (Fig. 2)
	−6‰	Sea water beneath ice in McMurdo Sound, south-western Ross Sea (Hole 3)[9]
	−250‰	Snow at Scott Base, McMurdo Sound‡

*δ value in parts per 10^3 deviation from Standard Mean Ocean Water (SMOW) (±3‰).
†All samples were vacuum distilled before analysis and were analysed using the hot probe method[8].
‡G. L. Lyon, personal communication.

ice cliff about 15 m high (Fig. 1); the northern margin is both less regular and more snow-covered than the southern margin. At the foot of one of the cliffs a narrow rift extends down to a thin ice floor with water beneath; chemical (Table 1) and isotopic (Table 2) analyses have shown that this is seawater.

The rift can be traced for more than 2 km along the foot of the ice cliffs, and it probably extends along their entire length. For a 50-m-long section of the rift (Fig. 2) the maximum width of the floor is 2 m, and its thickness is about 0.1 m. Several soundings through holes in the ice indicated an irregular underwater topography. The greatest depth plumbed at the eastern end of the rift was 2.9 m, and at the western end was 4.9 m. The 'feel' of the line indicated, however, that the surface was rough, hard and sloping, and not bottom. Soundings from a wider part of the rift should reach much greater depths.

The only sign of life along the rift is a yellowish brown patchy staining, which is common up to about 0.5 m above the water level on the northern side. The staining proved to be the remains of algae. Filamentous wisps as much as 80 mm long are abundant in the water under ledges and beneath the ice floor of the rift. Samples of the material include living organisms containing traces of fresh pigment (H. W. Johnstone, personal communication). The samples consist largely of the disrupted cell debris of centric diatoms, with some intact frustules, and a few whole pennalean diatoms. Disruption probably resulted from the freezing of the samples after collecting, for temperatures in the field ranged from −3 to −10 °C.

I had expected to be able to observe the tide, known to have a range of between 1.0 and 1.5 m over this part of the shelf[4], and

I placed a marker in the ice at sea level to check this (Figs 2 and 3). For the first 6 h the water level did not change more than 1 mm relative to the ice, but 24 h later the ice had risen 40 mm relative to the water level. Further markers were placed and measured on several occasions, though, following a blizzard, not all could be relocated for the final measurements.

The measurements are accurate to 2 mm, and establish two points. First, no tidal cycle is detectable from markers on either side of the rift. Because the tidal range is large enough to be measured directly it is concluded that the ice on both sides of the rift is floating (see Fig. 4). Second, the data also show that the rift zone is highly mobile. All four stations on the northern side, and two on the southern side, rose more or less steadily over a five day period at rates of between 38 and 65 mm d^{-1};the other two stations rose less than 29 mm over the five day period (Fig. 3). Two strain measurement triangles across the rift between stations 3 and 4 showed that the rift widened by 0.23 and 0.27 m over the 77-h period between 1000 on December 8 and 1500 on December 11, 1974. If these high rates of movement were maintained for more than a few tens of days the ice-floored rift would be many metres across and the margins of the rift would rise metres above the valley floor. Neither feature has, in fact, been observed along the rift, and it is therefore concluded that both the horizontal and the vertical movements form part of a long term 'glaciotectonic'

Fig. 2 Sketch plan and section of the rift containing seawater, showing locations of the eight pegs measured for Fig. 3.
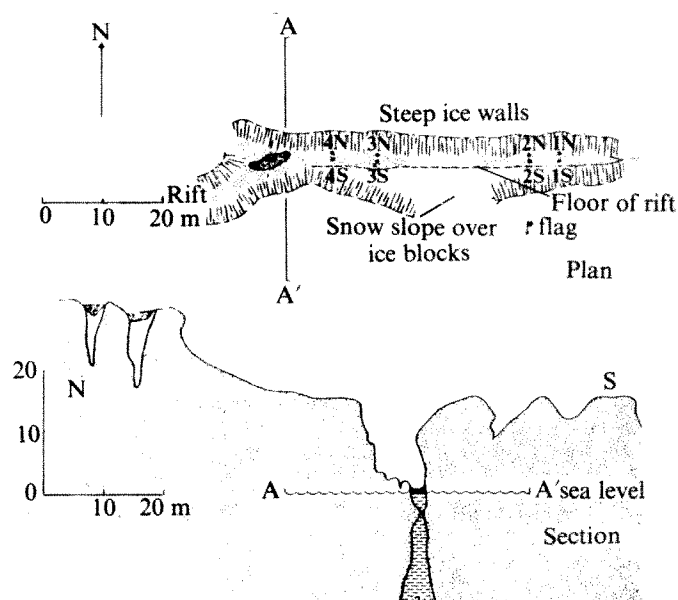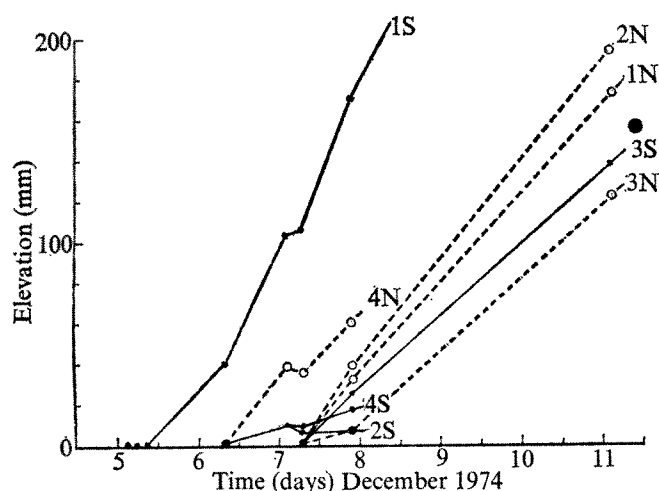
Fig. 3 Vertical movement of pegs on the north and south sides of the rift floor. Peg locations shown in Fig. 2. ●, South side; ○, north side.

cycle, with a period probably in the range 10–100 d. These loca' movements are believed to relate to the regional ice flow pattern, but further and more accurate measurements are necessary to check this.

I conclude that the rift is filled with water in free circulation with the Ross Sea. The salinity is slightly higher than Antarctic surface water or circumpolar deep water, but lies within the range of Ross Sea shelf water[5] (34.75 ‰ –> 35.00 ‰). Furthermore, the presence of living algae indicates a direct connection with the waters of the open Ross Sea 550 km to the north. The
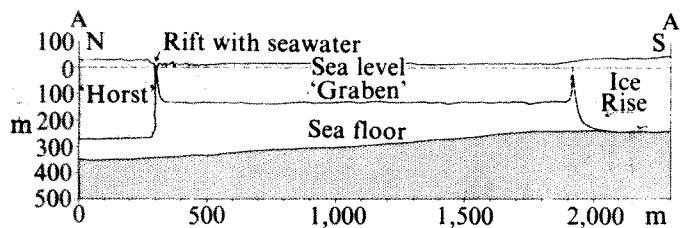
Fig. 4 Cross-section north from the edge of the ice rise to a block of the Ross Ice Shelf (A–A' in Fig. 1), showing underwater ice and bottom topography inferred from surface elevations with respect to sea level.

normal marine salinity indicates that there is little freezing or melting in the rift, or at least that the circulation is sufficient to prevent a measurable change in salinity. The mean annual temperature is −27 °C (ref. 6), but the temperature gradient, and thus the rate of freezing or melting, of ice forming the rift is not known.

The discovery of seawater near the head of the Ross Ice Shelf provides a topographic datum (sea level) that was not previously available in the region. The rift itself also provides biologists with access to an unusual new ecosystem. The fishing prospects are quite good too, for specimens of the benthic fish *Trematomus bernacchii* have recently been caught beneath permanent ice cover 100 km from the open sea in the Antarctic Peninsula[7]. Experience suggests that crevassed areas on ice shelves are worth closer examination. In the past they have been regarded as hazards to be avoided, but it is now evident that some crevasses have considerable scientific value as natural access ways to the sea beneath.

I thank Ross Powell, Rob Rainsbury and John Stevens for assistance and support in the field, and Jim Newman for help

Antarctic Research Centre,
Victoria University of Wellington,
Wellington,
New Zealand

[1] Zumberge, J. H., and Swithinback, C., *Am. geophys. Un. Antarct. Ser.*, 7, 197–208 (1962).
[2] Bentley, C. R., Clough, J. W., and Robertson, J. D., *Antarct. J. U.S.*, 9(4), 157–158 (1974).
[3] Robin, G. de Q., *Nature*, 253, 168–172 (1975).
[4] Robinson, E. S., Neuburg, H. A. C., and Williams, R., *Antarct. J. U.S.*, 9(4), 162–163 (1974).
[5] Jacobs, S. S., Amos, A. F., and Bruchhausen, P. M., *Deep Sea Res.*, 17, 935–962 (1970).
[6] Bentley, C. R., Cameron, R. L., Bull, C., Kojima, K., and Gow, A. J., *Antarctic Map Folio Ser.*, 2, Plate 5 (1964).
[7] Heywood, R. B., and Light, J. J., *Nature*, 254, 591–592 (1975).
[8] Thurston, W. M., *Rev. scient. Instrum.*, 42, 700 (1971).
[9] Gow, A. J., and Epstein, S., *J. geophys. Res.*, 77, 6552–65 (1972).

Cryptic suture in the eastern Grenville Province

IN order to explain palaeomagnetic data from the Precambrian Shield of North America (Laurentia), Irving *et al.*[1] have suggested that Grenvillia was separated from Interior Laurentia 1,150 Myr ago and then rejoined it about 1,000 Myr ago to form the present day Canadian Shield. This hypothesis requires that a suture exists within the Grenville Province south of a region of Grenville rocks which are considered to be metamorphosed equivalents of rocks within the adjacent, older structural provinces, and north of sampling sites used to determine Grenvillia poles (Fig. 1); the Grenville Front has been discounted as the site of the suture[1]. A weakness of this hypothesis is that there is no known geological evidence for a suture in the appropriate location. It has been suggested[1], however, that the crust now exposed as the Grenville Province originally occupied deep crustal levels (10–20 km) and that any traces of former intervening oceans may have been destroyed. We present here an interpretation of gravity data in the region which is in accord with the presence of a cryptic suture within, or proximal to, the eastern part of the suture zone as delineated by the palaeomagnetic investigations.

A Bouguer anomaly map of the eastern Grenville and neighbouring regions (Fig. 1) shows a striking, linear negative anomaly, the Grenville Front Low[2], extending from Lake Mistassini to the Labrador coast, a distance of about 1,200 km. For the whole of this distance the anomaly straddles the Grenville Front and its axis lies consistently within the Grenville Province. In profile (Fig. 2) the anomaly is asymmetrical with gradients on the northern flank consistently averaging about 0.35 mgal km⁻¹, and those on the southern flank maintaining an average of about 1 mgal km⁻¹. The linearity of the southern flank is locally disturbed by anomalies associated with large gabbroic intrusions. The background level of anomalies north of the Grenville Front Low is noticeably lower by about 15 mgal (ref. 2) than that over the Grenville Province.

Various hypotheses—massive granites[3], a metasedimentary trough[4], and a crustal root combined with low velocity–low density seismic layers[5]—have been advanced to explain the anomaly, but strong evidence in favour of one or other of these hypotheses is largely lacking[2]. The anomaly is clearly the expression of large scale, crustal structure and not a manifestation of discrete lithological variations. Tanner[6] has suggested a model comprising a denser than 'normal' Grenville upper crust in contact with Superior crust of 'normal' density; the remaining portions
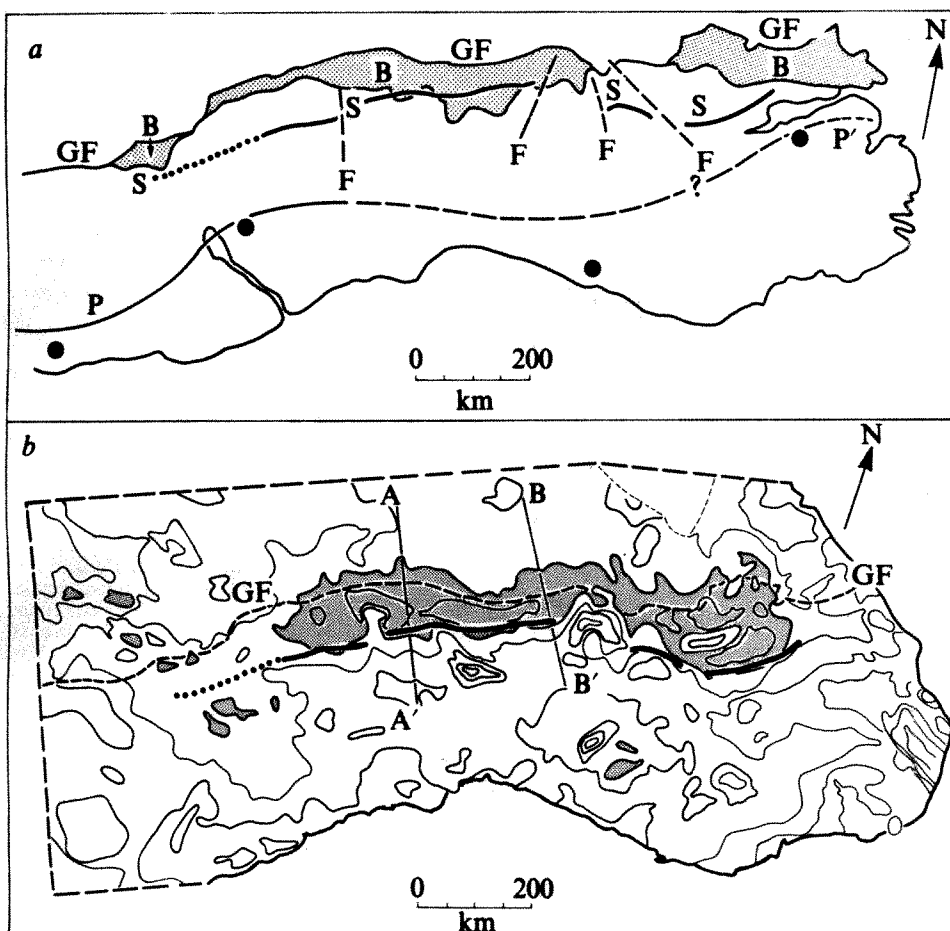
Fig. 1 *a*, Outline map of eastern Grenville Province: B (shaded areas), terrain B of Irving *et al.*[1]; GF, Grenville Front; P–P', northern limit of palaeomagnetic poles obtained for Grenvillia; dashed lines (F), interpreted faults; heavy solid lines (S), trace of suture interpreted from gravity anomalies; dotted line (S), trace of suture interpreted from magnetic anomalies; ●, sampling sites for palaeomagnetic sudies[1]. *b*, Bouguer anomaly map of eastern Grenville Province; contour interval, 20 mgal: shaded areas, anomalies with values of less than —80 mgal; position of suture outlined with same line pattern as in *a*; GF, Grenville Front; A–A', B–B', lines of gravity interpretations illustrated in Fig. 2.

of both crusts are of 'normal' density. On this model the compensating base of the thicker Grenville crust would be the principal cause of the low and of the gentle northern flank of the anomaly, and the denser upper crust, dipping about 45° south-eastwards from near the Grenville Front to a uniform depth of 20 km, would explain the steep southern flank and the higher background level of anomalies over the Grenville Province. The thicknesses of the Superior and Grenville crusts (35 and 40 km, respectively[6]) have been confirmed seismically[5].

Seismic work[5] has also indicated a two-layer crust for both structural provinces, with the Conrad discontinuity occurring at depths of 14 and 21 km, respectively, under the Superior and Grenville provinces; a thicker crust (45 km) with the Conrad discontinuity at a depth of 24 km was indicated under the axis of the Grenville Front Low. Using the seismic information Thomas[2] proposed a model in which the gentle northern flank of the anomaly results from the downward flexuring of the older structural provinces towards the axis of the anomaly. The effect of such downwarping is to induce lateral density changes between the upper and lower crusts and between the lower crust and the mantle, thus producing mass deficiencies. Supporting evidence for such flexuring is provided by the preservation of the string of Aphebian and Helikian sedimentary–volcanic basins along the northern side of the Grenville Front. The seismic root[5] may also contribute to the gravity low. The steep flank is formed by a major break separating a denser Grenville crust from Superior crust. This postulated break occurs up to 100 km south of the Grenville Front, thereby placing the front itself entirely within Superior-type crust. We suggest that this break is the line of the cryptic suture.

Gravity interpretations for a single layer crust along the lines A–A' and B–B' of Fig. 1 are shown in Fig. 2. The

models are extremely simple and in each case comprise Superior-type crust (34 km thick) in contact with Grenville crust (39 km thick) which is 0.06 g cm^{-3} denser; the density of the mantle is 0.4 g cm^{-3} greater than that of the Superior crust. Under the axis of the anomaly the Superior-type crust attains a thickness of 38 km, but this value falls short of the 45 km predicted from the seismic analysis[5]. It is impossible to reconcile gravity and seismic thicknesses without changing the density of the lower crust under the axis of the gravity anomaly. The contact between the two crustal blocks in both models slopes southwards under the Grenville Province, from points near the lower portion of the southern flank of the anomaly; these points are located 37 km (A–A') and 55 km (B–B') south of the Grenville Front. On the basis of these interpretations it has been possible to sketch the surface trace of the suture (Fig. 1) which is extended south-westwards along a line separating areas of strongly contrasting magnetic patterns[7].

Evidence of a major, continuous structural feature has not yet been recognised on the surface along the predicted trace. We believe, however, that this feature represents a cryptic suture of the type presented in the continental collision–basement reactivation model of Dewey and Burke[8] (such as the Indus Suture). The widespread distribution of anorthosites and granulite facies metamorphism within the Grenville Province is compatible with this model for the crustal levels exposed at present. At these levels it is likely that ultramylonites and pseudotachylites, which are believed to occur in the deeper levels of sutures[8], will ultimately be discovered in the region of the proposed suture. Dewey and Burke[8] believe that the analogue of the Indus Suture for the Grenville situation lies south-east of the exposed Grenville Province. Our model suggests, however, that suturing has occurred along the northern edge of the Grenville Province which concurs with the hypothesis
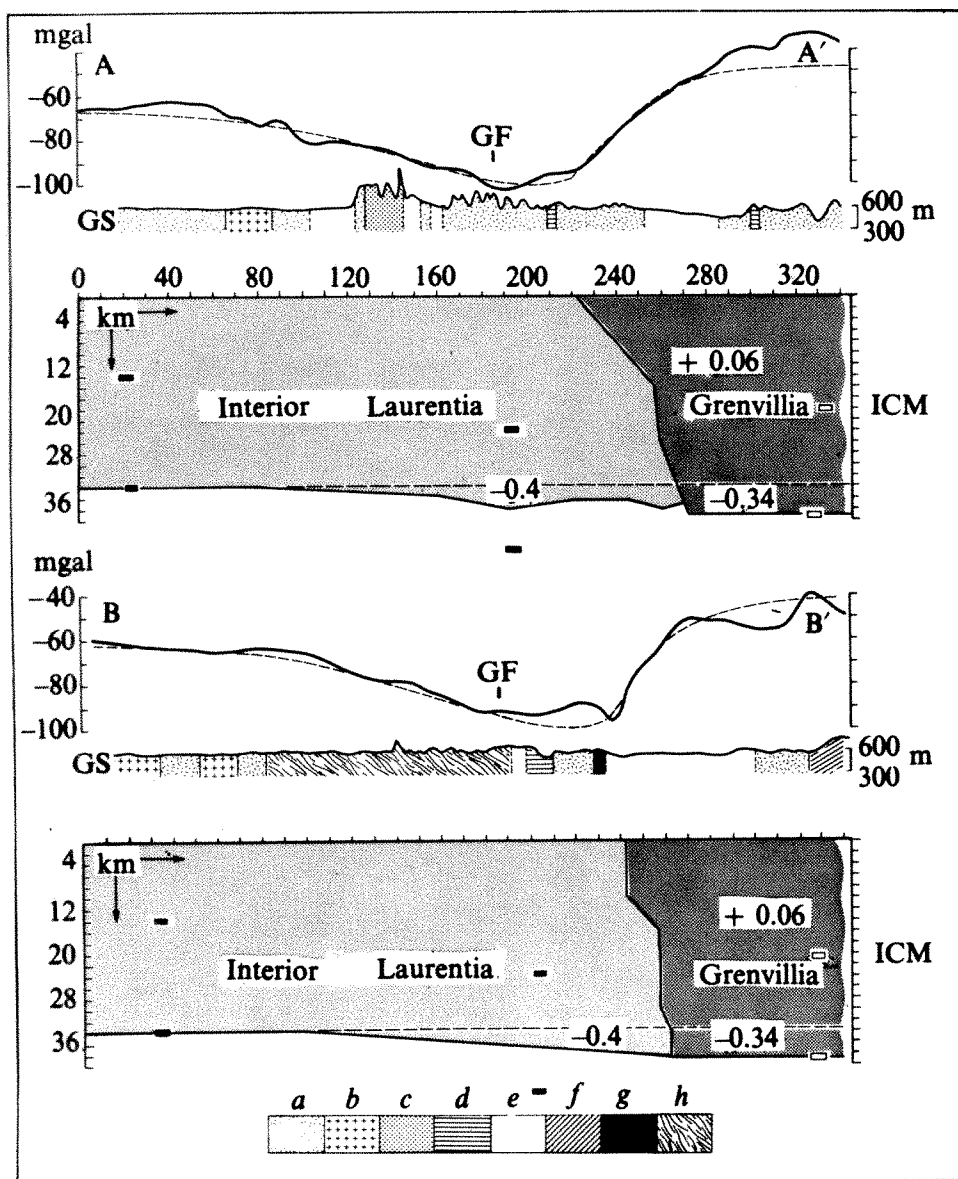
Fig. 2 Profiles of observed Bouguer anomalies (heavy solid lines) and calculated anomalies (light dashed lines), near-surface geological sections (GS), and interpreted crustal models (ICM) along lines A–A' and B–B' of Fig. 1; density contrasts $(g\,cm^{-3})$ are indicated for the interpreted crustal models. Horizontal bars, positions of the Conrad and Mohorovicic discontinuities (upper and lower, respectively) determined by seismic experiments (solid bars, true horizontal positions; hollow bars, extrapolated from outside the region); GF, Grenville Front. Lithological key: *a*, quartzofeldspathic gneiss; *b*, granite to granodiorite; *c*, Otish Mountains sediments; *d*, quartzite, dolomite and iron-formation; *e*, drift or unmapped; *f*, sillimanite-bearing gneiss; *g*, gabbro; *h*, pyroxene-bearing granodiorite and granodiorite.

of Irving *et al.*[1]. Subduction would have occurred in this case from north-west to south-east.

We thank especially Dr R. A. Gibb for discussion and comments, and Dr P. Kearey for suggestions.

M. D. THOMAS
J. G. TANNER

Gravity Division,
Earth Physics Branch,
Department of Energy Mines and Resources,
Ottawa, Canada K1A 0E4

1 Irving, E., Emslie, R. F., and Ueno, H., *J. geophys. Res.*, 79, 5491-5502 (1974).
2 Thomas, M. D., *Gravity Map Series* (Department of Energy, Mines and Resouces, Ottawa, 1974).
3 Innes, M. J. S., *Trans, Am. geophys. Un.* 38, 156-165 (1957).
4 Grant, F. S., *Can. J. Earth Sci.*, 5, 895-906 (1968).
5 Berry, M. J., and Fuchs, K., *Bull. seism. Soc. Am.*, 63, 1393-1432 (1973).
6 Tanner, J. G., thesis, Univ. Durham (1969).
7 *Geol. Surv. Can.*, Map 1255A (2nd ed.), (1971).
8 Dewey, J. F., and Burke, K. C. A., *J. Geol.*, 81, 683-692 (1973).

Design of organic metals and superconductors

I WISH to draw attention to the potential of odd alternant hydrocarbons (OAHs) in the design of organic metals and superconductors which gives reason to suppose that they may prove equal or even superior to present systems. There is now strong evidence for the existence of the metallic state in certain organic charge transfer salts, and a suggestion of low temperature superconducting fluctuations in the case of tetrathiofulvalene–tetracyanoquinodimethane (TTF–TCNQ); present interest is now focused on the stabilisation of these states at greater (critical) temperatures[1]. In contrast to the molecular crystals usually formed by conjugated organic molecules (which are held together by van der Waals' forces and characterised by a relatively weak intermolecular interaction), these salts crystallise as segregated one-dimensional stacks of cation and/or anion radicals in which the charge transfer process and thus the electronic mobility within a stack is greatly enhanced by the abnormally high overlap of the π-electron wave functions of nearest neighbours in the stack and by the ability of the constituent molecules to stabilise an ionic fluctuation.

Odd alternant hydrocarbons are described in refs 2 and 3; it will be sufficient to note that within the Huckel molecular orbital (HMO) theory, neutral OAHs possess one unpaired electron in a formally non-bonding molecular orbital, and as alternant hydrocarbons have zero charge density at all atoms. It also follows that an OAH can give rise to a stable, closed shell cation and anion (in which there is effective dispersal of charge density) and that the bond orders (and by inference bond lengths) remain invariant among this triad of oxidation states. Furthermore, the discussion will depend on the extrapolation of the properties of the isolated molecules into the crystalline state. This assumption is likely to provide a reasonable description of the solid state properties, as these one-dimensional

charge transfer compounds give rise to a single narrow conduction band (the bandwidth of which is proportional to the intermolecular electron-transfer integral (t)), and may be studied in the tight-binding approximation[1,4]. The conduction band is formed from a linear combination of the singly occupied molecular orbitals (conveniently termed conduction molecular orbitals (CMOs) on each molecule in the one-dimensional lattice. The systems are usually treated in terms of the Hubbard[5] Hamiltonian, and within this model the Hartree approximation indicates that the metal–insulator transition[6,7] will occur at $U_{eff}/4t = 1$, where U_{eff} is the energy of an ionic fluctuation $(..M...M...\rightarrow..M^+...M^-..)$ and t is the intermolecular transfer integral which arises from overlap between the CMOs on adjacent sites M (where $M = $ TTF$^+$, TCNQ$^-$ or OAH).

For comparison I choose the ionic charge transfer salt (TTF–TCNQ) (refs 1, 8 and 9), the CMOs of which are shown in Fig. 1 together with those of OAHs phenalenyl (PLY) (refs 3 and 10 and my unpublished work) *sym*-tribenzophenalenyl[11,12] and *sym*-nonabenzophenalenyl. The various criteria which are thought to be important in the design of organic metals and superconductors have been thoroughly discussed by others[1,13-15]; they are: (1) The existence of unpaired electrons. Neutral OAHs are free radicals and as such possess one unpaired electron in a non-degenerate MO (just as TTF$^+$ and TCNQ$^-$).

(2) A uniform crystal structure. Unlike TTF$^+$ and TCNQ$^-$, no counter-ion is required for OAHs which may form totally homogeneous crystals.

(3) Relatively weak electron–electron repulsion (minimisation of U_{eff}). SCF MO calculations (my unpublished results) show that PLY has an ionic fluctuation (or disproportionation) energy of the same order of magnitude as TTF$^+$ and TCNQ$^-$. This stems from the favourable dispersal of charge and the absence of di-ions in this process (that is, 2 OAH $\rightarrow$ OAH$^+$ + OAH$^-$; compare with 2 TCNQ$^-$ $\rightarrow$ TCNQ + TCNQ^{2-}) in this latter respect OAHs are more like normal (Group IA and IB) metals. This is particularly dramatic when it is remembered that PLY is a hydrocarbon, and a further reduction in the ionic fluctuation energy would be expected in systems which are appropriately substituted at the active positions, and in the larger OAHs where there is an even greater delocalisation of charge density.

(4) Maximisation of the intermolecular transfer integral (t). This may be accomplished in two basic ways: First, by maximisation of the degree of overlap between the CMOs of adjacent molecules in the stack; second, by reducing the intermolecular spacing. As shown below, OAHs may be expected to prove superior to ionic charge transfer salts on both counts.

The TCNQ$^-$ ions in charge transfer salts are known to occur in a variety of columnar stacking arrangements[16-20], but in none of these are the TCNQ$^-$ ions directly over each other, so as to give optimal overlap of the CMOs. In the highly conducting salts, such as (TTF–TCNQ) (ref. 20), the ions stack to give overlap patterns of the CMOs which are essentially translated by one node in the wave-functions, and probably correspond to the second best phase relationship between adjacent molecules. The non-optimal stacking arrangement in charge transfer salts may be attributed to a combination of two factors: the need to maximise the coulomb attraction with counter-ions[21] (see (2) above), and the unfavourable intracolumnar electrostatic interaction between the high charge densities which are present in these ions. This repulsion is maximised in a completely superimposed stacking arrangement (even in the offset configuration the interaction is high, see below). This contrasts with the case of OAHs which are neutral radicals, and where the local charge densities are extremely small (zero in the HMO approximation). It is therefore to be expected that OAHs will adopt a totally eclipsed stacking arrangement with optimum overlap of the CMOs, particularly if the unpaired electrons in charge transfer compounds make a significant contribution to the cohesive binding of the crystal[21] (as in
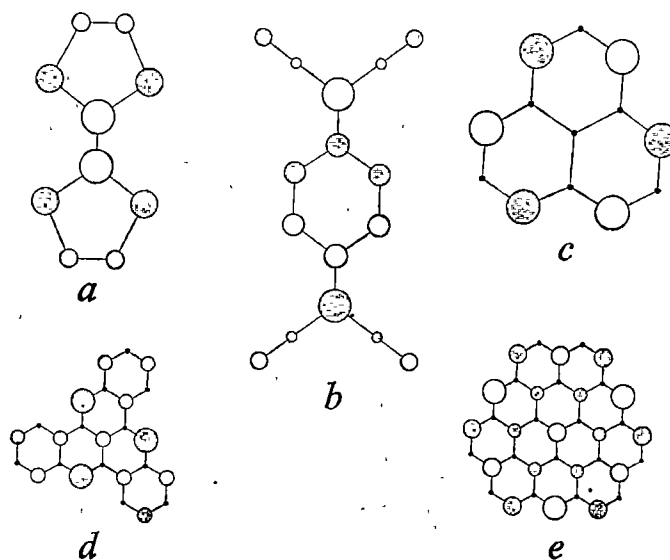
Fig. 1 The CMOs of TTF$^+$ (*a*), TCNQ$^-$ (*b*), PLY (*c*), *sym*-tribenzophenalenyl (*d*), and *sym*-nonabenzophenalenyl (*e*). The CMO is the highest (singly) occupied MO in the molecule and forms the basis for the conduction band in one-dimensional face-to-face charge transfer compounds. The dimensions and wave functions of (*a*)–(*c*) were calculated by SCF MO theory, whereas (*d*) and (*e*) are shown with HMO wave functions and standard geometries[2]. Open and stippled circles represent the positive and negative coefficient, respectively, of the $2p\pi$ atomic orbital; ., denotes a node in the wave function.

normal metals).

Turning now to the second point, the reduction of the intermolecular spacing in charge transfer compounds, it is appropriate to begin by considering the Madelung constant (α) of a one-dimensional lattice of positive or negative point charges (which are assumed to represent TTF$^+$ or TCNQ$^-$). The result is the same in both cases, and the Madelung constant is given by $\alpha = 2(1 + \frac{1}{2} + \frac{1}{3} - \frac{1}{4} + ...)$. This series is divergent, and as the magnitude of the repulsion between adjacent sites is quite large (a value of 3.0 eV has been estimated[22] for TCNQ$^-$ ions), it is apparent that the intermolecular approach between adjacent molecules in salts such as TTF$^+$ and TCNQ$^-$ is limited by this unfavourable electrostatic contribution to the cohesive binding energy of the crystal along the one-dimensional axis. Of course OAHs, being neutral molecules, have a Madelung constant $\alpha = 0$. With the removal of the coulombic repulsion along the one-dimensional axis, decreased intermolecular spacing would be expected to follow. Furthermore, the intermolecular spacing between adjacent TCNQ$^-$ molecules has been shown to exert a profound effect on the electronic structure of these solids, and it is significant that the salt with the highest conductivity so far [1,8,9] has the closest approach between adjacent TCNQ$^-$ ions[20]. This is not surprising as t is of exponential form, and at the characteristic separation shows great sensitivity to distance. For example, a decrease in the intermolecular separation of 0.2 Å from the "normal" value of 3.2 Å (TCNQ$^-$) leads to an increase in t of more than 30% (my unpublished work).

(5) The question of superconductivity in organic metals is now in a state of flux (see refs 1, 9, 13–15, 23–29). A full discussion of the relevance of OAHs to this general area will be deferred to a later paper; the likely differences in the phonon spectra of OAHs and conventional systems such as TTF and TCNQ suggest that OAHs hold promise in the design of organic superconductors. In the parent OAHs the coupling between the intramolecular vibrational levels and the conduction electrons is minimal (zero in the HMO approximation), as the CMOs are formally non-bonding and the occupancy of these orbitals therefore makes no contribution to the bond order. This contrasts with systems such as TTF and TCNQ, where in the case of TCNQ electron addition progressively

changes the molecular character from quinonoid to benzenoid with accompanying nuclear reorganisation. This effect (which in the crystal may be viewed as the intramolecular phonon contribution to the polaron binding energy) has been implicated in both the high conductivity[1,25] (as a mechanism for electron-pair formation), and in the non-metallic transition observed for (TTF–TCNQ). In the latter case it has been suggested[30-33] that the librational distortion provides the driving force for a Peierls transition[34]; clearly this behaviour would be inhibited in OAHs where, in the case of PLY, I calculate the distortion energy to be decreased by at least an order of magnitude. Furthermore, if the intramolecular contribution to the transverse optical branch of the phonon spectrum does make a contribution to the high conductivity of salts such (TTF–TCNQ)[1,25], an analogous effect may be introduced into OAHs in an easily controllable manner by exocyclic substitution at the active positions. Finally it is appropriate to point out that ionic fluctuations in charge transfer compounds will always be strongly coupled to the longitudinal acoustic branch of the phonon spectrum and in the case of OAHs will lead to a shortening of the intermolecular spacing (with concomitant increase in the transfer integral) due to the formation of an adjacent cation–anion pair, which should be particularly favourable to the collective mode involved in Frohlich-type superconductivity[23,24,35] and may even stimulate cooperative polaron formation between chains.

Of the OAHs, PLY is probably best known[3], and there is an extensive chemistry for cation, neutral radical (there is some tendency towards dimerisation in solution[3,10]), and anion; nevertheless, one of the chief attractions of OAHs is the diversity of structures and substitution patterns which may be chosen from, in order to satisfy the design specifications of organic metals and superconductors.

ROBERT C. HADDON

Research School of Chemistry,
Australian National University,
PO Box 4 Canberra, ACT 2600, Australia

1 Garito, A. F., and Heeger, A. J., *Accts chem. Res.*, 7, 232 (1974).
2 Dewar, M. J. S., *The Molecular Orbital Theory of Organic Chemistry* (McGraw-Hill, New York, 1969).
3 Reid, D. H., *Q. Rev. chem. Soc.*, 19, 274 (1965).
4 Epstein, A. J., Etemad, S., Garito, A. F., and Heeger, A. J., *Solid State Commun* , 9, 1803 (1971); *Phys. Rev.*, B5, 952 (1972).
5 Hubbard, J., *Proc. R. Soc.*, A276, 238 (1963); A277, 237 (1963); A281, 401 (1964).
6 Mott, N. F., *Proc. phys. Soc.*, A62, 419 (1949).
7 Austin, I. G., and Mott, N. F., *Science*, 168, 71 (1970).
8 Ferraris, J., Cowan, D. O., Walatka, V., and Perlstein, J. H., *J. Am. chem Soc* , 95, 948 (1973).
9 Coleman, L. B., et al., *Solid State Commun.*, 12, 1125 (1973).
10 Reid, D. H., *Tetrahedron*, 3, 339 (1958).
11 Clar, E., and Stewart, D. G., *J. chem. Soc* , 23 (1958).
12 Clar, E., Kemp, W. and Stewart, D. G., *Tetrahedron*, 3, 325 (1958).
13 Special issue of *J. Polym. Sci. C.*, 29, (1970).
14 Shchegolev, I. F., *Phys. Stat. Sol. (a)*, 12, 9 (1972)
15 Yagubskii, E. B., and Khidekel, M. L., *Russ. Chem. Rev.*, 41, 1011 (1972)
16 Herbstein, F. H., in *Perspectives in Structural Chemistry*, IV (edit. by Dunitz, J. D., and Ibers, J A.), 166 (Academic, New York, 1971).
17 Kobayashi, H., Ohashi, Y., Marumo, F., and Saito, Y., *Acta Cryst.*, B26, 459 (1970).
18 Hoekstra, A., Spoelder, T., and Vos, A., *Acta Cryst.*, B28, 14 (1972).
19 Sundaresan, T., and Wallwork, S. C., *Acta Cryst.*, B28, 491 (1972).
20 Phillips, T. E., Kistenmacher, T. J., Ferraris, J P., and Cowan, D. O , *Chem. Commun.*, 471 (1973).
21 Metzger, R M., *J. chem Phys.*, 57, 1870, 1876, 2218 (1972).
22 Hiroma, S., Kuroda, H., and Akamatu, H., *Bull. chem. Soc. Jap* , 44, 9 (1971).
23 Bardeen, J., *Solid State Commun.*, 13, 357 (1973).
24 Allender, D., Bray, J. W., and Bardeen, J., *Phys. Rev. B*, 9, 119 (1974).
25 Gutfreund, H., Horovitz, B., and Weger, M., *J. Phys C.*, 7, 383 (1974); *Phys. Rev. B*, 9, 1246 (1974); *Solid State Commun.*, 15, 849 (1974).
26 Bari, R. A., *Phys. Rev. B* , 7, 2128 (1973).
27 Coll, C. F., and Beni, G., *Solid State Commun.*, 15, 997 (1974).
28 Lee, P. A., Rice, T. M., and Anderson, P. W., *Phys. Rev. Lett.*, 31, 462 (1973).
29 Fukuyama, H., Rice, T. M., and Varma, C. M., *Phys. Rev. Lett.*, 33, 305 (1974).
30 Bright, A. A., Garito, A. F., and Heeger, A. J., *Solid State Commun.*, 13, 943 (1973).
31 Morawitz, H., *Bull. Am. phys. Soc.*, 18, 1577 (1973).
32 Grant, P. M., Greene, R. L., Wrighton, G. C., and Castro, G., *Phys. Rev. Lett.*, 31, 1578 (1973).
33 Perov, P. I., and Fischer, J. E., *Phys. Rev. Lett.*, 33, 521 (1974).
34 Peierls, R. E., *Quantum Theory of Solids* (Oxford University Press, London, 1955).
35 Frohlich, H., *Proc. R. Soc.*, A223, 296 (1954).

Recent climatic warming around New Zealand

MANY papers have described the climatic fortunes of areas in the Northern Hemisphere over the past few hundred years; the progressive warming during 1890–1940, and the present deterioration. Some people have suggested that the globe could be descending into an ice age (see ref. 1), but such assumptions are derived mainly from data for the Northern Hemisphere. Moreover, data for the Southern Hemisphere used[2,3] so far have chiefly been obtained from locations between the Equator and 40 °S. Information from higher latitudes, where any variations are amplified, is sparse. Here we present the results of an examination of a small area in the mid-latitudes of the Southern Hemisphere which has been warming over the past thirty years.

New Zealand is a long narrow country which straddles the lattitudes 34°–47°S, with a SW–NE oriented axial range, the Southern Alps, rising to 3,764 m in the South Island. The southern westerly wind belt which crosses southern New Zealand gives higher precipitation on the windward west coast, and less on the leeward east, whereas the north of the country protrudes into the subtropical high pressure belt.

Extensive records of temperature, rainfall and air pressure for ten locations (Fig. 1) were analysed: six urban, two rural, and two small islands[4,5]. All stations had minimal site changes except Auckland where temperature correction factors were applied. The data were investigated using 5-yr (short term variation) and 20-yr (longer term trends) running means. The rural stations were used to check that changes seen in urban areas were not attributable to the

Fig. 1 Location diagram of all sites in the New Zealand region quoted in this report. Hatched area indicates land above 2,000 m.
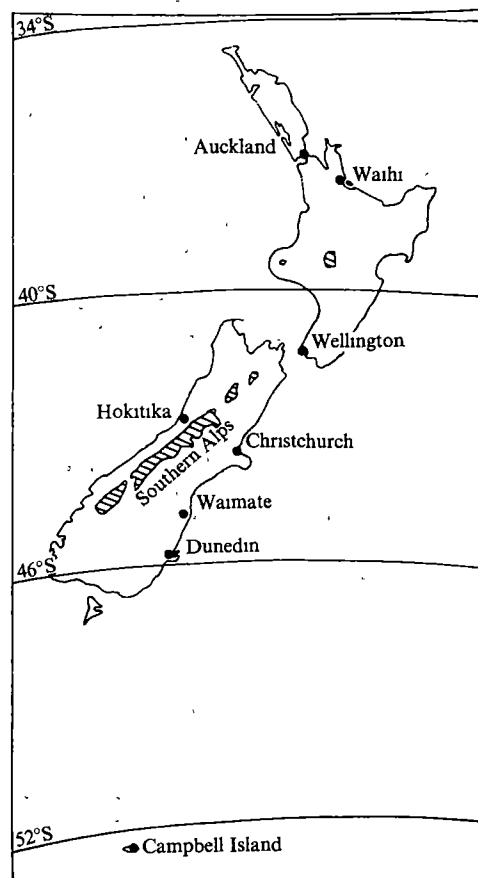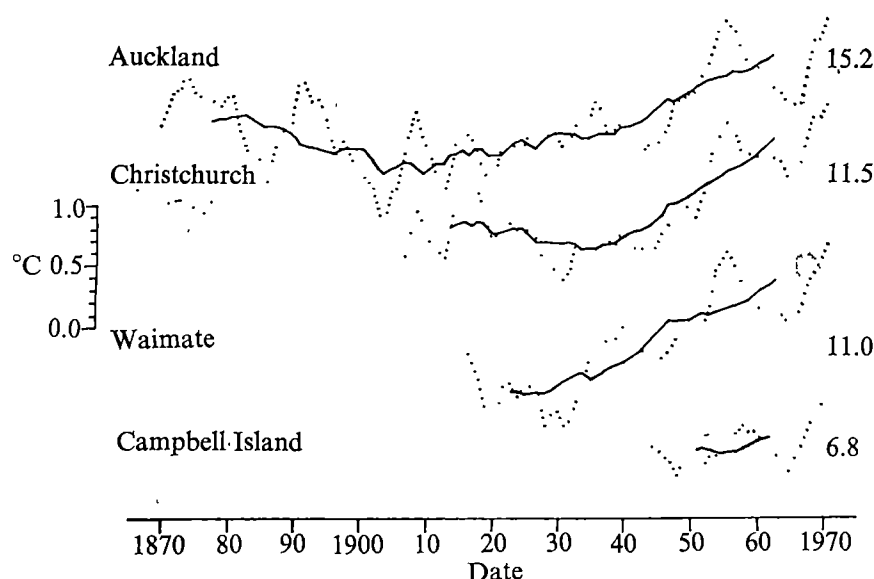
Fig. 2 ——, Twenty-year running means of mean annual temperature showing trends typical of New Zealand locations; . . ., five-year running means. Temperature scale shown diagrammatically at left. Numbers indicate average annual mean temperature of the entire station record. Breaks in graphs indicate incomplete records.

heat island effect found in cities. Auckland barometric pressure acted as a position indicator for the subtropical anticyclonic belt. The Auckland–Dunedin pressure differences was used as an index of the strength of the westerlies over New Zealand.

Figure 2 shows typical temperature fluctuations and variations in the New Zealand area. Fluctuations over the whole region are in phase, though of different amplitudes. The urban heat island effect does not interfere with the

now the area is enjoying its warmest spell since temperature measurements began. The warmest single year yet recorded over all New Zealand was 1971.

Rainfall analysis over New Zealand shows no significant temporal variation. Air pressure analysis (Fig. 3) reveals higher pressure over the country around the early 1890s when weak westerlies occurred The subtropical ridge has migrated north gradually since 1894 accompanied by a great strengthening of the southern westerlies over New
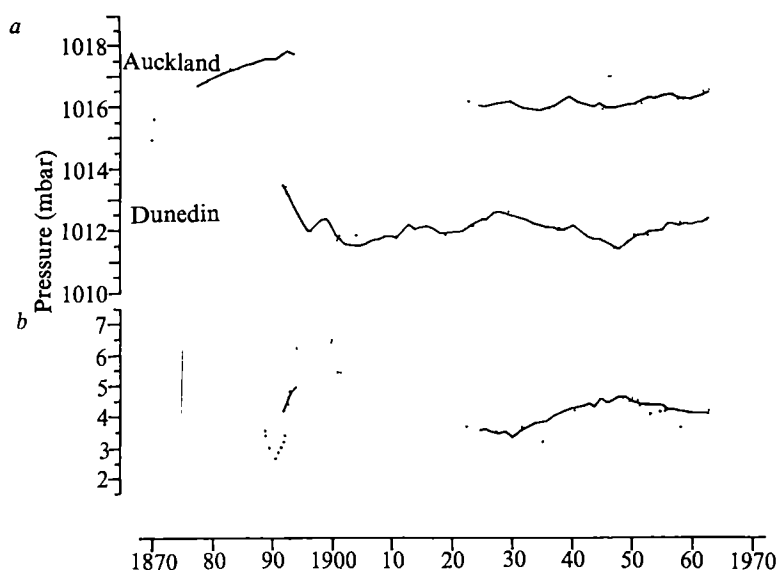
Fig. 3 *a*, Running means of mean annual air pressure at Auckland and Dunedin; *b*, running means of the Auckland–Dunedin air pressure gradient. Notation as in Fig. 2.

trends observed; Waimate, a rural centre 180 km from Christchurch, has variations almost identical to those of urban Christchurch; Waihi and Auckland respond similarly.

The climatic vicissitudes show no great fluctuations in the initial record up to 1880 (Fig. 2). Climatic deterioration occurred in the two decades 1880–1900, the temperatures being cool about 1885, and mild around 1893, but with a declining trend over the whole of New Zealand. The period 1900–1935 was the coldest over the country in recorded history with the years about 1904, 1913, 1921 and 1931 being especially harsh. Climatic amelioration has been experienced throughout the whole region during 1935–1970, temperatures climbing by 1 °C over these years. The mid-1950s were very much warmer than average and

Zealand by 1898, and strong westerlies persisted for the next 10 yr. The migration of the subtropical ridge, with strengthening of the westerlies could well explain the climatic deterioration experienced. Auckland barometric pressure has risen slowly since records began again in 1918 indicating a slow and continual southward shift of the subtropical anticyclonic belt over the past 50 yr. The westerlies over New Zealand weakened around 1935, the time when the present amelioration began. Apart from a small strengthening of the westerlies around 1946 when a small downward fluctuation in the rising temperature trend occurred, the westerlies have not been strong. The southerly migration of the high pressure zone and weaker westerlies over New Zealand seems to explain the recent warming.

Our results reveal that climatic changes in the New Zealand area do not parallel published curves for the globe[2,3], so we must re-examine the validity of climatic trends for the Southern Hemisphere computed with little regard to data from points south of the 40th parallel. New Zealand was cooling in the period 1880–1900, and had its coldest period during 1900–1935 when the Northern Hemisphere was experiencing some of the warmest years on record[4]. Conversely, in the past three decades the New Zealand area has warmed up while northern continents have been cooling rapidly. The five year running means of Scott Base, Antarctica, show a rise from −21 °C in 1960 to −15.9 °C in 1969 (ref. 5). Of eight main Australian urban centres examined from statistical publications for individual Australian states[6-8], seven show at least a 0.5 °C rise since 1945. Orcadas Island at latitude 60° 45' S near South America, similarly has warmed[1] by 0.5 °C since 1940. So it seems that the warming in the New Zealand region is common to a wider range in the Southern Hemisphere.

M. J. SALINGER

Department of Physiology,
University of Otago Medical School,
Dunedin, New Zealand

J. M. GUNN

Department of Geography,
University of Otago,
Dunedin, New Zealand

1 Keyzer, R., *New Zealand Listener*, 77, 13-15 (1975).
2 Mitchell, J. M. Jr, *Ann. NY Acad. Sci.*, 95, 235-250 (1961).
3 Mitchell, J. M. Jr, *Quat. Res. (NY)*, 2, 436-445 (1972).
4 *Statistics of New Zealand, 1860-1927* (New Zealand Government Printer, Wellington), (Registrar General's Office; subsequently Government Statistician; subsequently Census and Statistics Department; now Department of Statistics).
5 New Zealand Meteorological Service, *Meteorological Observations, 1928-1973*, (New Zealand Government Printer, Wellington).
6 World Weather Records, *Smithsonian Misc. Colls.*, 79, (1944).
7 *Ibid.* 90, (1944).
8 *Ibid*, 105, (1947).

Solid state batteries containing silver sulphonium iodide electrolyte

THE high ionic conductivity α phase of AgI can be stabilised at room temperature by the addition of various inorganic or organic salts (such as Ag_4RbI_5, ref. 1) to the AgI. These systems have attracted considerable interest as electrolytes in solid state batteries[2,3].

We have investigated the properties of electrolytes based on silver iodide with various sulphonium iodides, and we have shown that the solids so prepared are good ionic conductors. Conductivities of pressed pellets were measured as a function of the molar ratio of the components and the composition corresponding to the maximum conductivity was used in subsequent formulations. Examples of the systems studied are $AgI-(CH)_3SI$, $AgI-(CH_2)_4SCH_3I$, $AgI-O(C_2H_4)_2SCH_3I$, $AgI-(CH_2)_5SCH_3I$ (ref. 4) for which the maximum conductivities fall in the range 3×10^{-3} $(\Omega\, cm)^{-1}$ to 4×10^{-2} $(\Omega\, cm)^{-1}$. We are now investigating these and related electrolytes in solid state batteries; we have shown that battery systems can be prepared which have good voltage–time characteristics, good mass efficiency and in which the electrolyte is compatible with the electrode material (thus indicating potentially good shelf life). Such a battery has the characteristics of a commercially viable system.

$Ag_7(CH_2)_4SCH_3I_8$ electrolyte has been used in a typical battery. The anode is composed of Ag powder, electrolyte and graphite or carbon black. It was found that mass efficiency was increased by using silver powder of low particle size (Johnson Matthey Cypher 88 silver powder, 90% by weight having particle size less than 5 μm).

The cathode, which in these examples is functioning as a source of iodine, is prepared by reacting a sulphonium iodide with iodine to form a sulphonium polyiodide which is then mixed with electrolyte and graphite. In practice, only $(CH_2)_4$-SCH_3I_3 (melting point 89 °C) and $(CH_2)_4SCH_3I_5$ (melting point 54 °C) of the cheap sulphonium polyiodides are useful because the melting points of the others are too low to be of practical use.

The anode, electrolyte and cathode powders are pressed together into a compacted pellet which forms the battery in the subsequent tests. For reasons of compatibility, the system chosen for the initial tests had the same sulphonium iodide in both the electrolyte and in the cathode.

With this system, using $(CH_2)_4SCH_3I$ as the sulphonium iodide, the electrolyte was unstable in the presence of polyiodides higher than the tri-iodide. The evidence for this was:

(1) The resistances of pressed pellets of mixtures of $Ag_7(CH_2)_4$ SCH_3I_8 + iodine, $Ag_7(CH_2)_4SCH_3I_8$ + $(CH_2)_4SCH_3I_5$ and $Ag_7(CH_2)_4SCH_3I_8$ + $(CH_2)_4SCH_3I_3$ were taken at various intervals at 37 °C. The change (Fig. 1) in resistance indicated the following reactions:

$$Ag_7(CH_2)_4SCH_3I_8 + I_2 \xrightarrow{\text{fast}} 7AgI + (CH_2)_4SCH_3I_3 \quad (1)$$
$$\text{(resistance rose sharply)}$$

$$Ag_7(CH_2)_4SCH_3I_8 + (CH_2)_4SCH_3I_5 \xrightarrow{\text{slow}}$$
$$7AgI + 2(CH_2)_4SCH_3I_3 \quad (2)$$
$$\text{(resistance rose slowly)}$$

$$Ag_7(CH_2)_4SCH_3I_8 + (CH_2)_4SCH_3I_3 \longrightarrow \text{no reaction} \quad (3)$$
$$\text{(no change in resistance)}$$
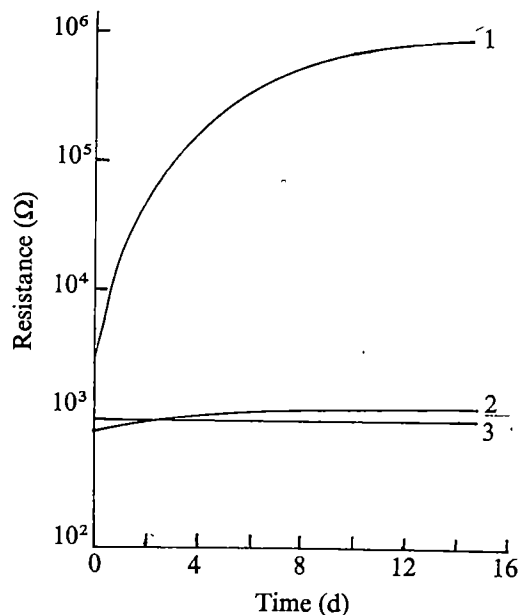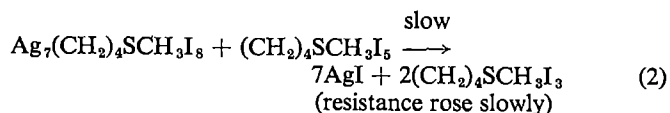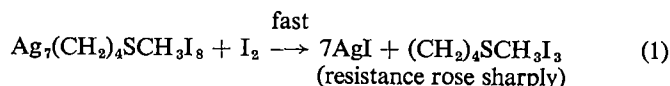
Fig. 1 Time variation of resistance, at 37 °C, illustrating results on which equations (1), (2) and (3) are based.

(2) Batteries at 37 °C using $(CH_2)_4SCH_3I_3$ in the cathode showed good voltage time characteristics whereas $(CH_2)_4SCH_3I_5$ in the cathode showed poor voltage time characteristics (Fig. 2).
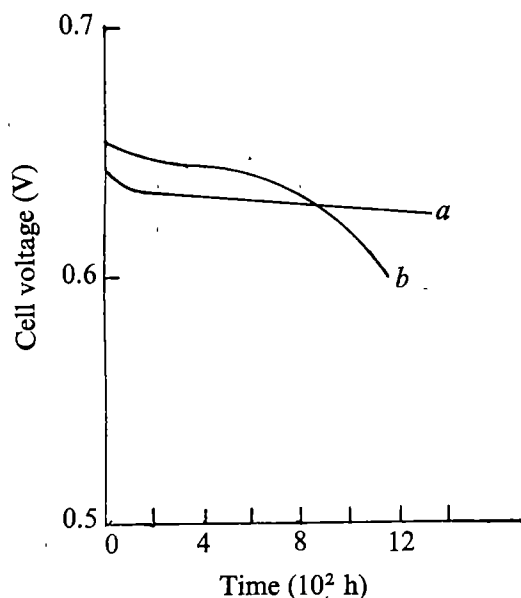
Fig. 2 Time variation of cell voltage, at 37 °C, for cathodes containing: *a*, $(CH_2)_4SCH_3I_3$; *b*, $(CH_2)_4SCH_3I_5$.

(3) When the batteries were cut open at the end of the test the electrolyte with $(CH_2)_4SCH_3I_3$ in the cathode still remained a pale yellow colour whereas the electrolyte with $(CH_2)_4SCH_3I_5$ in the cathode had reacted according to the second equation and was much darker in colour.

We thank Mallory Batteries (UK) Ltd for support. Further details will be reported elsewhere.

R. G. LINFORD
J. M. POLLOCK
C. F. RANDELL

School of Chemistry,
Leicester Polytechnic,
Leicester, UK

1 Raleigh, D O , *J. appl. Phys.*, 41, 1876–1877 (1970).

2 Owens, B B , *Fast Ion Transport in Solids*, 593–606 (North Holland, 1973)

3 Liang, C. C., *Appl. Solid State Sci.*, 4, 95–135 (Academic, New York and London, 1974).

4 *British Provisional Patent Application* No. 51848 (November, 1974).

Heavy metal binding sites in river water

THE transport of heavy metals (HMs) and their effects on biological systems in natural waters are greatly influenced by their interactions with soluble and insoluble water components[1,2].

We have found that ion-specific electrodes can serve as a convenient, precise and sensitive means of measuring the binding ability of solutions for a number of HM ions. We have studied the binding strengths and binding sites of river water for Hg^{2+}, Pb^{2+}, Cd^{2+}, and Cu^{2+} ions, using specific electrodes (see also ref. 3). We used standard membrane filters to retain particles (0.45 μm pore size) as have been used in past studies[4,5]. We also found that the use of standard ultrafilters to retain macro-molecules (in these studies, of molecular weights 45,000, 16,000 and 1,400) may give a much finer definition of the size of binding sites than those obtained previously by filtration studies. These techniques may prove to be very valuable in

studying the dynamics of heavy metal transport and utilisation in natural waters. They could probably be automated for *in situ* determinations of chelation capacity of such waters.

The Hg^{2+} binding capacity (expressed as α, the fraction of the added ion which is bound) of a sample of water from the Ottawa River before and after filtration through different sized ultrafilters is shown in Fig. 1. The ion was strongly bound until 15 p.p.m. had been added; then the bound fraction decreased rapidly, indicating that saturation of the binding capacity of the water had been achieved. Passage of the water through filters, even those retaining molecules with a molecular weight of 1,400 had little or no effect on binding capacity.

The binding capacity of other heavy metal cations showed a different response to filtration. With Pb^{2+} ions, for example, saturation of the metal-binding capacity of water was spread over a much wider range of added HM ions (Fig. 2). Thus, an equilibrium could exist with substantial amounts of free Pb^{2+} ions. Passage of the water through a 0.45 μm filter did not reduce Pb^{2+} binding capacity, but passage through smaller filters significantly reduced it. Some of the Pb^{2+} binding components were less than 0.45 μm in diameter but had molecular weights greater than 45,000. Others had molecular weights between 45,000 and 16,000, and still others had molecular weights of less than 1,400.

The binding capacity of river water for all the ions, and the equilibrium binding constants of these ions with river water are presented in Tables 1–3, which also show the changes in binding capacity for different HM ions caused by the passage through different sized filters. Cu^{2+} ions behaved like Pb^{2+} ions, except that substantial amounts of Cu^{2+} binding activity were removed after passage through a 0.45-μm filter; that is, as shown earlier for other water samples[4], a good deal of Cu^{2+} binding activity is particulate. The low Cd^{2+} binding ability was unchanged by filtration through any but the smallest filters.

Fig. 1 Binding of Hg^{2+} by river water. α, The bound fraction, is calculated as $(T_M - [M^{2+}])/T_M$ where T_M is the total HM^{2+} added and $[M^{2+}]$ is the concentration of free HM^{2+} determined by the specific ion electrode. Increments of $Hg(NO_3)_2$ were added to 50-ml samples of Ottawa River water, unfiltered or passed through membrane filters or ultrafilters: *a*, 45,000 molecular weight filter; *b*, unfiltered and 0 45-μm filter; *c*, 16,000 and 1,400 molecular weight filter.

Total Hg^{2+} ion (p.p.m.)
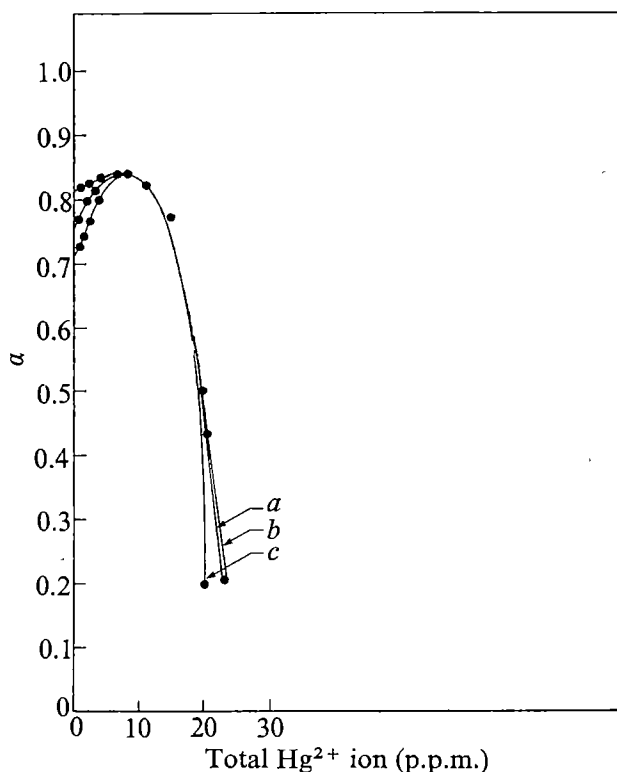
Table 1 Binding properties of Ottawa River water

Conditional constants* for M^{2+}–river water		Equilibrium constants for M^{2+}–fulvic acid complexes	Metal-holding capacity (p.p.m.) of river water	Calculated value for [river component]‡
K_{Hg}	$1.25 \pm 0.46 \times 10^6$	$Hg^{2+} \approx 5.41 \times 10^{11}$	$Hg^{2+} \approx$ 11 p.p.m. at < 15 p.p.m. of T_{Hg}^{2+}	5.5×10^{-5} M
K_{Pb}	$8.88 \pm 3.8 \times 10^3$	$Pb^{2+} \approx 4.35 \times 10^3$	4 p.p.m. at > 15 p.p.m. of T_{Hg}^{2+}	2.0×10^{-5} M
K_{Cu}	$5.01 \pm 1.58 \times 10^3$	$Cu^{2+} \approx 2.85 \times 10^3$	$Pb^{2+} \approx$ 4 p p.m.	1.9×10^{-5} M
K_{Cd}	$4.69 \pm 0.9 \times 10^3$	$Cd^{2+} \approx 1.81 \times 10^3$	$Cu^{2+} \approx$ 1.6 p.p.m.	2.54×10^{-5} M
			$Cd^{2+} \approx$ 0.6 p.p m.	5.4×10^{-6} M

Mean [river component]§ $2.15 \pm 0.2 \times 10^{-5}$ M

*Constants calculated from $[M^{2+}]$ measured by ion-specific electrodes.
+Fulvic acid concentration = 10^{-3} M.
‡Calculated as $[M^{2+}]_{bound}$ at equilibrium conditions.
§Mean value calculated excluding value of Hg^{2+} at < 15 p.p.m. T_{Hg}^{2+}.

The chemical nature of the HM binding sites in river water is unknown, but we have shown that the binding is not dependent on the presence of HCO_3^- or CO_3^{2-} ions in Ottawa River water. When the water was acidified, gassed with N_2 to drive off CO_2 and neutralised, the binding ability for all HM ions remained the same. In contrast, water from other bodies of water lost a good deal of binding activity after this treatment, implying that HCO_3^- or CO_3^{2-} ions play an important part in such binding. These ions have been implicated in the binding of Cu^{2+} and Cd^{2+} by water from rivers in England[4,5]. We considered the possibility that other inorganic anions might be involved in HM binding. The level of phosphate in this water (about 1 part per 10^9) was too low to be significant in HM binding. The limit of detection of sulphate was about 5 p.p.m.; if present, such levels could be significant. When samples of ultrafiltered (molecular weight, 1,400) Ottawa River water were evaporated to dryness, ashed (at 900 °C for 16 h) and reconstituted with deionised water, all the HM binding activity was lost. Separate experiments showed that such treatment did not reduce the HM-binding ability of pure Na_2SO_4 solution. We know of no other inorganic anions likely to be present in significant amounts. The ultrafiltered water is free from suspended particles or macromolecules, and so binding must be a result of the formation of chemical complexes rather than of physical adsorption on surfaces. We think it very probable that the complexing components are organic molecules, destroyed or volatilised during ashing.

Table 2 Total loss (%) in metal-binding capacity after ultrafiltration

	Filter types			
M^{2+}	0.45 μm	45,000 m.w.	16,000 m.w.	1,400 m.w.
Hg^{2+}	0	0	11.7	11.7
Pb^{2+}	0	31.9	47.9	56.4
Cu^{2+}	34.4	82.6	82.6	82.6
Cd^{2+}	0	0	0	46

Table 3 Metal-binding capacity (p.p.m.) of ultrafiltered (molecular weight 1,400) river water before and after removal of inorganic and organic carbon compounds

M^{2+}	Untreated	Inorganic carbon removed	Total carbon removed
Hg^{2+}	11.0	11.04	0.6 p.p.m.
Pb^{2+}	4.0	4.0	No binding
Cu^{2+}	1.6	1.7	0.075 p.p.m.
Cd^{2+}	0.6	0.55	No binding

Fig. 2 Binding of Pb^{2+} by river water. Calculations as in Fig. 1. $Pb(NO_3)_2$ was added to river water samples. *a*, Unfiltered and 0.45 μm filter; *b*, 16,000 m.w. filter; *c*, 45,000 m.w. filter; *d*, 1,400 m.w. filter.

The possibility that fulvic acid, probably the most important soluble 'humic substance' in natural waters[6-8], played a major part in HM binding was investigated by comparing the binding pattern of a sample of fulvic acid isolated from Podzol B_h soil[8] with the binding pattern of Ottawa River water towards different HM ions. Quite a different pattern was obtained: a 10 p.p.m. solution of fulvic acid had about the same binding ability towards Hg^{2+} ions as did river water, but a much weaker ability to bind the other heavy metal ions than did filtered or unfiltered river water samples. A comparison of the equilibrium constants of Ottawa River water and fulvic acid with the different HM ions (Tables 1-3) shows that fulvic acid cannot account for the HM binding capacity of this water. Studies on the HM binding pattern of fulvic and humic acids isolated from natural waters could help in deciding the role of these complex groups of substances in metal binding and transport by water systems.

A more detailed description of this work and information on the HM binding of other bodies of water has been submitted

for publication elsewhere. The work was supported by the Ottawa River Project, a joint study by the National Research Council of Canada Laboratories and the University of Ottawa.

S. RAMAMOORTHY
D. J. KUSHNER

Department of Biology,
University of Ottawa,
Ottawa K1N 6N5, Canada

1 Gibbs, R. J., Science, 180, 71-73 (1973).
2 Ong, H. L., Swanson, V. E., and Bisque, R. E., Prof. Pap. U.S. geol. Surv., 700C, C130-37 (1970).
3 Ramamoorthy, S., and Kushner, D. J., Microbial Ecology (in the press).
4 Stiff, M. J., Wat. Res. 5, 585-599 (1971).
5 Gardiner, J., Wat. Res. 8, 23-30 (1974).
6 Packham, R. F., Wat. Treat. Exam., 13, 316-329 (1964).
7 Schnitzer, M., Proc. Soil Sci. Soc. Am. 33, 75-81 (1969).
8 Schnitzer, M., and Khan S. U., Humic Substances in the Environment (Marcel Dekker, New York, 1972).

Controlled growth of crystalline silicate fibres

DURING studies of the crystallisation kinetics of aluminosilicate glasses of approximately metasilicate composition, surface nucleated crystals have been observed to grow in a fibrous habit[1]. This has been attributed to the presence of β-CaSiO$_3$, the low-temperature polymorph of calcium metasilicate, the mineral equivalent of which, wollastonite, occurs naturally in a similar form.

To produce β-CaSiO$_3$ fibres in a more controlled fashion it is necessary to increase crystal growth rates in these glasses so that a zone heat treatment can be applied. An investigation by hot stage microscopy of the crystallisation kinetics of various compositions in the CaSiO$_3$ phase region has revealed that certain ternary glasses possess satisfactory growth-rate/temperature characteristics for this purpose. A glass of composition CaO–SiO$_2$–ZnO (30:50:20 weight %) was chosen as the most suitable material for initial experiments as it can be drawn easily into vitreous rods of about 1 mm diameter.

Attempts to induce longitudinal crystallisation in these rods by moving them slowly past an annular heating element proved ineffective as not only did crystals nucleate from the surface but the heated zone also slumped considerably, thus forming a brittle and distorted product. But if the rod is passed through an electrically heated platinum coil supporting a molten zone, any such crystals are removed, and β-CaSiO$_3$ crystallises from the supercooled melt on the far side in a truly aligned and fibrous habit. Since the glass does not pass through a low temperature before crystallising, neither surface nor internal nucleation can occur spontaneously.

The essential features of this experimental arrangement are shown in Fig. 1, together with an estimated longitudinal temperature profile and the corresponding growth rate profiles. Crystallisation is initiated by introducing a platinum probe into the melt at the start of the process, and thereafter the crystal front is self-stabilising at a position and temperaure where its growth rate is equal to the speed of travel of the rod. The temperature of crystallisation in a particular rod thus depends solely on its speed of travel, whereas any variation in heating power to the molten zone (which must be above the liquidus of the glass) merely changes the shape of the temperature profile and therefore the position of the crystal front. Since the glass composition is such that the α/β-CaSiO$_3$ inversion temperature is situated on the growth rate curve above the temperature peak, speeds below about 20 µm s^{-1} will cause the high-temperature polymorph α-CaSiO$_3$ to crystallise, whereas speeds greater than the growth rate maximum of about 50 µm s^{-1} will produce a non-crystalline glass rod.

Examination of thin sections of rod by optical microscopy reveals that β-CaSiO$_3$ crystallises as an array of parallel fibres some 5 µm in diameter right through the rod, so that only a few percent of residual glass remains. Laue X-ray patterns confirm a very high degree of orientation, with the crystallographic b-axis (which corresponds to the chain direction of the SiO$_4$ tetrahedra) aligned longitudinally. There is, in addition, some orientation in the plane perpendicular to the fibre axis, apparent in scanning electron micrographs of fracture surfaces (Fig. 2). In contrast, α-CaSiO$_3$ grown from the same glass composition but at lower speeds exhibits very little fibrous character or fracture behaviour, and the Laue pattern shows no recognisable orientation of its Si$_3$O$_9$ ring structure.

Rods of α-CaSiO$_3$ which are 1 mm in diameter have a rupture strength of 320 MN m^{-2} and an elastic modulus of 91 GN m^{-2}. These values are virtually identical to those of the base glass; but β-CaSiO$_3$ rods show significantly higher values of 540 MN m^{-2} and 124 GN m^{-2}, respectively. By comparison, rods prepared by conventional methods, which contain fairly random orientations of surface nucleated and internally nucleated crystals are too delicate to allow three-point bend tests to be carried out.
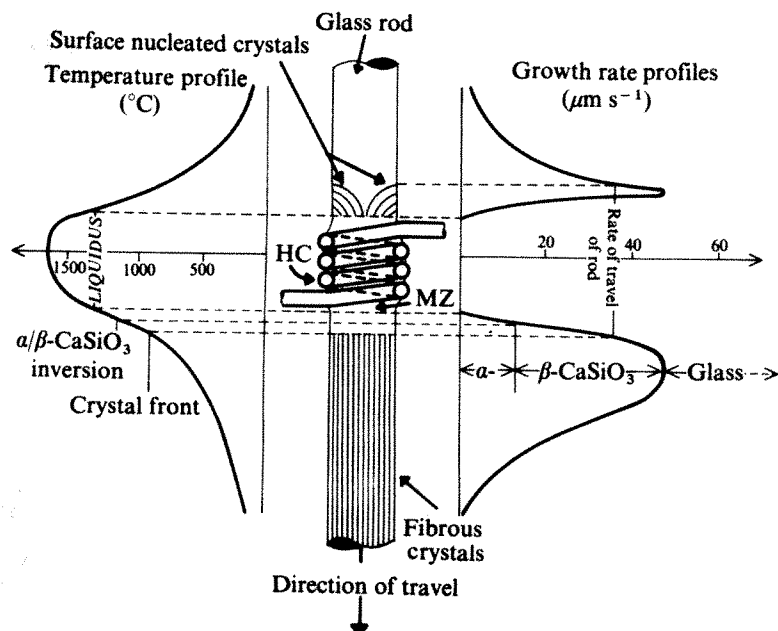
Fig. 1 Schematic diagram of apparatus for crystallisation from the melt (not to scale). The longitudinal temperature distribution, difficult to measure directly, is inferred to be approximately exponential, and corresponding growth rate curves are drawn from hot-stage micrography data. HC, Heating coil; MZ, molten zone.
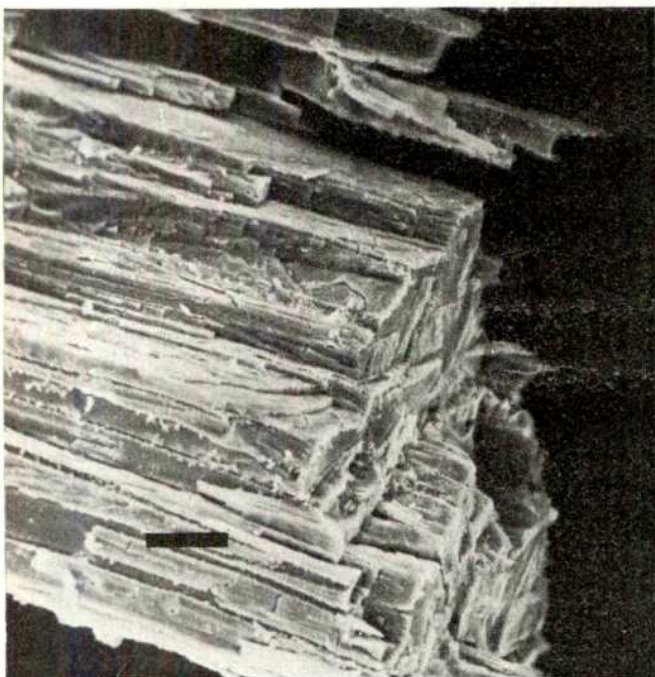
Fig. 2 Scanning electron micrograph of β-CaSiO₃ rod fractured in three-point loading (longitudinal view). Scale bar: 20 µm.

This novel technique of directional devitrification from a molten zone may be extended both to produce aligned crystal growth in rods with larger cross sections and to draw continuous filaments directly from molten glass compositions which will crystallise at higher rates. Furthermore, it could be applied to any silicate or other system which crystallises in a sheet or chain structure.

A. MARIES
P. S. ROGERS

Nuffield Research Group,
Department of Metallurgy and Materials Science,
Imperial College, London SW7 2BP, UK

¹ Williamson, J., Tipple, A. J., and Rogers, P. S., *J. Iron Steel Inst.*, **206**, 899–903 (1968).

New primitive therian from the early Cretaceous of Mongolia

THERIAN mammals with tribosphenic molars were probably in existence at the beginning of the Cretaceous[1]. This conclusion is based on a single heavily worn lower molar from the Lower Wealden (Neocomian) of England, named *Aegialodon dawsoni*. The molars of the Jurassic therians, (Pantotheria and Symmetrodonta) were capable only of puncturing and shearing. Tribosphenic molars have an additional cusp on the upper molars (protocone) which fits into a basin (talonid) on the matching lower molar. Unfortunately, the single lower molar of *Aegialodon* is extensively worn and slightly damaged. Consequently, it is difficult to discuss with confidence the structure of the earliest known tribosphenic molars, or the inferred structure of the matching upper molars, or the occlusal relationships. In this paper a brief description is given of an early Cretaceous mammalian lower molar from the Aptian of Asia. It is almost identical to that of *Aegialodon*. Because it is exceptionally well preserved and practically unworn, it provides critical information on the structure and function of the very early tribosphenic molars.

The Khovboor beds in Guchin Us somon (county) in the province (aymak) of Arvaykher contain a fauna of early

Cretaceous mammals. Collections from this site (Laboratory of Stratigraphy and Palaeontology, Geological Institute, Academy of Sciences in Ulan Bator) consist of numerous isolated teeth and fragmentary mandibles of triconodonts, multituberculates and therian mammals, as well as the isolated lower molar described here. About seven tons of sediments from the same locality have been washed and screened[2], and the large collection of mammals obtained is housed in the Palaeontological Institute of the USSR Academy of Sciences in Moscow (currently being investigated by Dr B. A. Trofimov). Four new mammalian genera have already been named but not described[3]: *Gobioconodon borissiaki* Trofimov, *Gobiodon infinitus* Trofimov, *Prokennalestes kozlovi* Trofimov and *Prozalambdalestes simpsoni* Trofimov. The classification line is: Theria of metatherian–eutherian grade; Family Aegialodontidae; *Kielantherium* gen. nov. Dashzeveg; *Kielantherium gobiensis* sp. nov. Dashzeveg (*Kielantherium* is a monotypic genus, the generic diagnosis is the same as for the type species), (Figs 1 and 2).

Holotype: Right lower molar, possibly M₂—GI (Ulan Bator) No. PST 10–14.

Horizon and type locality: Lower Cretaceous (possibly Aptian), Khovboor, Guchin Us somon, Arvaykher aymak, Mongolian People's Republic.

Derivation of name: *Kielantherium*—named in honour of Professor Zofia Kielan-Jaworoska; *therion* Gr., an animal; *gobiensis*, from the Gobi Desert.

Material: The type is a lower molar and is the only representative of this new genus and species in the collection. In the collection in Moscow a mandible with two lower molars, PIN No. 3101–32 seems to be conspecific with the type. The Moscow specimen was not named or cited in the list of species from Khovboor[3].

Diagnosis: Lower molar with a relatively narrow talonid, a small hypoconid and hypoconulid and a poorly differentiated ridge supporting two or three crenulations along its medial border, but a distinct ectoconid is not present. The protoconid was considerably higher than paraconid and the latter only slightly higher than the metaconid. Paraconid and metaconid were clearly separated at their bases on the medial face of the tooth.

Kielantherium is assigned to the Aegialodontidae on the basis of a strong similarity to the lower molar of *Aegialodon* in that: the trigonid is small and narrow; shearing surfaces 5 and 3 are small[4]; both anterobuccal and anterolingual cusps are present; relative height of the principal trigonid cusps are the same in both genera; there is a clear separa-

Fig. 1 *Kielantherium gobiensis* gen. et sp. nov. Outline drawings of lower M₂(?). *a*, Crown; *b*, exterior; *c*, interior; *d*, anterior; *e*, posterior views.
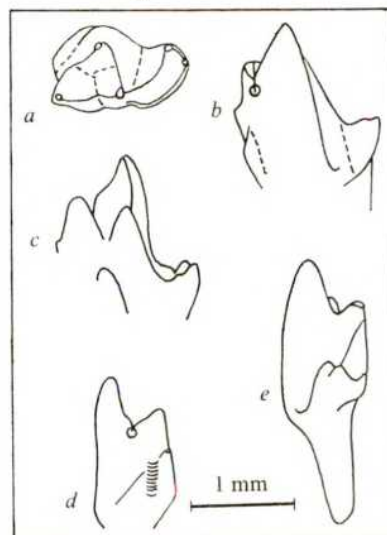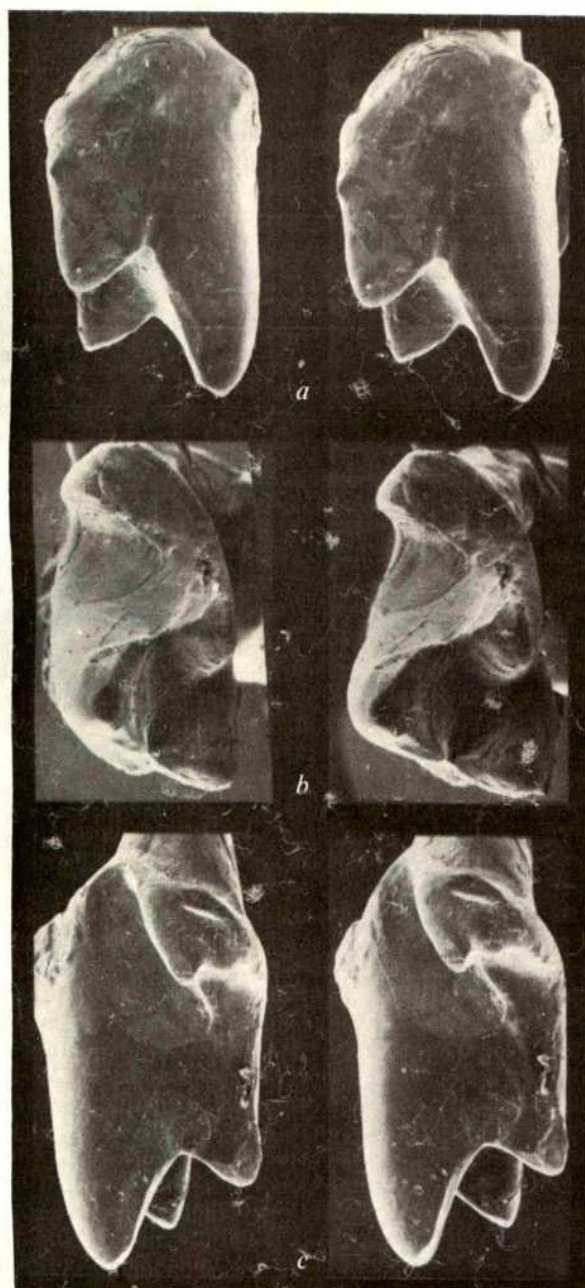
Fig. 2 *Kielantherium gobiensis* gen. et sp. nov. Stereophotographs. *a*, Posterior; *b*, crown; *c*, anterior of lower $M_2(?)$. $\times 36$.

tion between the bases of the paraconid and metaconid on the medial face of the tooth and; facet 1 can be divided into two subfacets by a line running parallel to the crista obliqua. *Aegialodon* and *Kielantherium* are remarkably similar to *Kermackia texana*[5] which is based on a single lower molar. The question which obviously arises is whether the type of *Kielantherium* is a premolar rather than a molar. The well developed trigonid cusps, the acute angle between the protocristid and paracristid and the referred specimen with two teeth *in situ* in the lower jaw in the collection from the Khovboor beds housed in Moscow, seem to rule out this possibility.

Kielantherium and *Kermackia* seem to be slightly younger in age than *Aegialodon* and are found in association with more advanced therians; *Prokennalestes* in the case of the former and *Pappotherium* and *Holoclemensia*[5] in the case of the latter. *Kermackia* and *Kielantherium* therefore seem to be survivors of mammals which had only recently developed a protocone in late Jurassic or early Cretaceous. This suggests that mammals with this feature may have had a wide distribution during the early part of the Cretaceous

(*Kermackia* in North America, *Aegialodon* in Europe, and *Kielantherium* in Asia).

Kielantherium differs from the late Cretaceous Deltheridiidae of Mongolia, *Deltatheridium* and Deltatheroides[6-8], in having a much more primitive structure, with a relatively smaller talonid, smaller talonid basin, in possessing an antero-lingual cusp which is absent in the Deltatheridiidae and in smaller dimensions.

I thank Professor A. W. Crompton for reading the manuscript and for helping in its preparation, Dr B. A. Trofimov for permission to study the collection of Khovboor mammals in his charge, and Professor Zofia Kielan-Jaworowska for the loan of the manuscript of her paper.

DEMBERELYIN DASHZEVEG

Laboratory of Stratigraphy and Paleontology,
Geological Institute, Academy of Sciences,
Ulan Bator, Mongolian People's Republic

1 Kermack, K. A., Lees, P. M., and Mussett, F., *Proc. R. Soc.*, B162, 535 (1965).
2 Kramarenko, N. N., *Trudy, Moskva*, 1, 9 (1974).
3 Beliayeva, E. I., Trofimov, B. A., and Resetov, V., Jr, *Trudy, Moskva*, 1, 19 (1974).
4 Crompton, A. W., *Early Mammals*, 65 (Academic, London, 1971).
5 Slaughter, B. H., *Early Mammals*, 131 (Academic, London, 1971).
6 Gregory, W. K., and Simpson, G. G., *Am. Mus. Novit.*, 225, 1 (1926).
7 Butler, P. M., and Kielan-Jaworowska, Z., *Nature*, 245, 105 (1973).
8 Kielan-Jaworowska, Z., *Paleont. Pol.*, 33 (in the press).

Cerebral dominance and reading habits

FREQUENTLY when an Israeli provides travel information in Hebrew, he points in one direction while simultaneously naming its opposite, for example he points to the left while saying: "You must turn right". When this contradictory behaviour is brought to his attention, he excuses himself and either points in the direction he had verbally indicated or corrects his error by changing the verbal expression to correspond with the physical. Observation of such verbal–spatial dissociation prompted the following experiments to explore systematically the possibility that Israelis make more errors in response to questions relating to left–right orientation than do non-Israelis.

Experiment 1 was a screening test. Four hundred right-handed college and high-school students (age range 16–27) participated. Two hundred were native-born Israelis; two hundred were new immigrants to Israel who had arrived within the past three years from Europe and North or South America. Each subject was asked, in random order and in his native language, to respond to a single command: "Look to the left" or "Look to the right." Eye movement responses were recorded by the examiner. Examiners were not informed of the purpose of the test.

Of the 200 new immigrants, 194 (97%) responded immediately and correctly; 6 (3%) responded with hesitation and with an initial movement of the eyes in the wrong direction (4 to the right, 2 to the left), but with subsequent self-correction. By contrast, only 128 (64%) of the 200 Israelis responded immediately and correctly, whereas 72 (36%) responded with hesitation and error (39 to the left; 33 to the right). The difference between native-born Israelis and new immigrants was highly significant ($z > 14.0$, $P < 0.001$; test for z value of significance of difference between proportions). Two types of error were noted among Israelis: 50 subjects (25% of the total Israeli group) responded with hesitation and with initial movement of the eyes in the wrong direction, followed by self-correction; 22 subjects (11%) looked in the wrong direction and did not self-correct. They did correct their direction of gaze, however, when the examiner repeated the command.

This screening test was repeated, in Arabic, with a group of 40 right-handed Arab students (age range 17–32). Twenty-three of the 40 subjects (57.5%) responded immediately and correctly; 17 (42.5%) responded with hesitation and with an initial movement of the eyes in the wrong direction (10 to the left, 7 to the right). Fourteen corrected

their errors without advice from the examiner; 3 did not correct their direction of gaze until the examiner repeated the question.

Experiment 2, a more demanding test of right–left orientation, was presented to 60 right-handed college students. Thirty were native-born Israelis (15 men, 15 women, age range 18–27, mean 21). Thirty were new immigrants to Israel who had arrived within the past three years from various European, North American and South American countries (15 men, 15 women, age range 18–26, mean 22). The test, developed for this experiment, required that the subject respond to an oral command by pointing to parts of the body, either on himself or on the examiner, as the examiner and subject sat facing each other.

Four types of questions were asked. In the 'self-uncrossed' condition, the subject had to point to a part of his own body without crossing the midline (for example, "With your right hand point to your right knee"). In the 'self-crossed' condition, the subject had to point to a part of his own body, across the midline (for example, "With your left hand, point to your right ear"). In the 'examiner-uncrossed' condition, the subject had to point to a part of the examiner's body, without crossing the midline of his own body (for example, "With your right hand, point to my left shoulder"). In the 'examiner-crossed' condition, the subject had to point to a part of the examiner's body by crossing the midline of his own body (for example, "With your right hand, point to my right eye"). Forty commands were given; ten for each condition. Half of the commands involved left hand and half right hand; commands were counterbalanced in each of the four conditions. The forty commands were presented in a predetermined, unpredictable order, and in the native language of each subject by examiners whose mother tongue corresponded with that of the subject. Examiners were not told of the purpose of the study until all testing was completed.

There was a striking difference in performance between native-born Israelis and new immigrants. Of a total number of 1,200 trials per group (30 subjects × 40 commands), Israelis made a total of 336 errors, whereas new immigrants made a total of 61 errors ($t=13.46$, $P<0.0001$; Student's t test).

Each of the sixty subjects made at least one error. No errors were made in the 'self uncrossed' condition. Most errors were made when subjects had to point to parts of the examiner's body and errors were more frequent in the crossed condition (see Table 1). Significantly more errors were made by the right hand than by the left hand, and by men than by women. These observations were similar for both Israeli and non-Israeli groups. Hesitation before response was more frequent in Israelis (23) than in non-Israelis (11).

These results show that native-born Israelis and Arabs

have significantly more difficulty with right–left orientation in response to oral verbal commands than do Europeans and Americans matched for age and educational level. These striking differences may be due to the influence of early-learned reading habits on cerebral function. I propose that reading habits of Europeans and Americans facilitate interhemispheric integration for initiating combined, verbal–spatial activity, whereas reading habits of Israelis and Arabs interfere with this integration.

Israelis and Arabs read from right to left; and Europeans and Americans read from left to right. These different patterns of reading are associated with different patterns of hemispheric activity. When Europeans and Americans start to read a word or line, both hemispheres are activated : the right hemisphere is activated for conjugate deviation of the eyes to the left; the left hemisphere is activated for verbal comprehension. When Israelis and Arabs start to read a word or line, the left hemisphere is activated both for conjugate deviation of the eyes to the right and for verbal comprehension.

Kinsbourne has proposed an attentional model of cerebral organisation which links orientation behaviour (as manifested by direction of gaze) to hemispheric laterality of thought process[1]. Thus, when silent verbal problem solving is being carried out, the eyes turn to the right, presumably because of activation of the left (language dominant) hemisphere. When silent visuospatial problem solving is being carried out, the eyes turn to the left, presumably because of activation of the right (spatial dominant) hemisphere. He further suggested that the physiological principle of reciprocal innervation[2] may apply not only at the spinal cord level but also at the level of the cerebral hemispheres. Accordingly, as one hemisphere is activated in the process of carrying out its functions, the other is inhibited with respect to its own major functions.

Applying these concepts to what was stated above concerning reading habits, we may conclude that initiation of reading for Europeans and Americans involves bilateral cerebral hemispheric activation, and concurrent bilateral hemispheric inhibition. For Israelis and Arabs the onset of reading involves activation of the left hemisphere, with concurrent inhibition of the right hemisphere. When repeated during a period of many years, the act of initiating the reading process gradually fosters the development in Europeans and Americans of bilateral interhemispheric integration for the initiation of tasks which require the simultaneous action of both hemispheres. This is not true, however, for Israelis and Arabs.

Thus, in the current experiments, in which an oral verbal command involving left hemispheric activation required a spatial response involving right hemispheric activation, the initial response of the European and American was more likely to be correct because the two cerebral hemispheres of these individuals had been educated to respond in an integrated manner at the beginning of such combined activity, whereas the initial response of the Israelis and Arabs was more likely to be slower or incorrect, because their brains require more time to allow interhemispheric integration to overcome the effects of the influence of reading habits.

I thank Drs H. Gordon and L. K. Obler for advice. Dr Phyllis Albert clarified the concepts and translated the paper into English.

MARTIN L. ALBERT*

Table 1 Performance on forty questions of left–right orientation

	Israeli ($n=30$)	Non-Israeli ($n=30$)
Errors		
Total	336	61
Examiner crossed	162	30
Examiner uncrossed	127	27
Self crossed	47	4
Self uncrossed	0	0
% of all questions	28	5
Mean per subject (s.d.)	11 (3.5)	2 (1.3)
Range	4–18	1–5
Hand making error		
Right	189	35
Left	147	26
Sex differences		
Errors by males	180	36
Errors by females	156	25

Department of Neurology,
Hadassah University Hospital,
Jerusalem, Israel

1 Kinsbourne, M., *Acta Psychol.*, 33, 193–201 (1970); *Science*, 176, 539–541 (1972); *Neuropsychology*, 12, 279–281 (1974).
2 Sherrington, C., *Integrative Action of the Nervous System* (Yale University Press, New Haven, 1906).

Electrophoretic differences between sympatric ecotypes

ELECTROPHORESIS has been used extensively for characterising genetic variability within and between natural populations[1,2] but has not been applied to sympatric ecotypes. In the mosquito *Aedes aegypti* a dark feral form predominates throughout sub-Saharan Africa, but elsewhere in the tropics and moist subtropics a paler domestic form is found. The two ecotypes occur together in Africa in areas with a dry season during which natives store water in pots inside huts[3,4]. The indoor ecotype breeds continually in water stored in human dwellings, and the outdoor ecotype lives in treeholes and other sites around the dwellings and

The starch-gel electrophoresis methods of Ayala[5] were used except as noted in the tables.

Gene frequencies for six loci are listed in Tables 1-3. The indoor and outdoor ecotypes differed considerably at three loci. In alkaline phosphatase, the indoor ecotype was fixed whereas outdoor strains had considerable genetic variation. For Coomassie blue (a protein stain), however, the reverse was true—the outdoor ecotype was completely or nearly fixed whereas the indoor ecotype was quite variable genetically. In the outdoor ecotype leucine aminopeptidase bands 0.54 and 0.59 were similar in amount of staining, whereas in the indoor ecotype band 0.59 stains heavily and band 0.54 is usually absent or obliterated by band 0.59. Band 0.59 is nearly always present, and its obliteration of 0.54 made counting frequencies of 0.54 impossible.

Table 1 Gene frequencies for esterase, alkaline phosphatase, Coomassie blue and malic enzyme

	Esterase			Alkaline phosphatase			Coomassie blue			Malic enzyme		
Allele position	0.90	0.86	n	0.69	0.66	n	0.27	0.24	n	1.00	0.88	n
Indoor												
Rabai	1.00	0	82	1.00	0	98	0.16	0.84	89	1.00	0	57
Moyo	1.00	0	98	1.00	0	98	0.29	0.71	87	1.00	0	53
Mgandini*	0.99	0.01	95	1.00	0	95	0.08	0.92	95	1.00	0	90
Outdoor												
Rabai	1.00	0	83	0.75	0.25	101	1.00	0	101	1.00	0	46
Kombeni	1.00	0	97	0.82	0.18	93	1.00	0	87	1.00	0	44
Kombeni*	0.96	0.04	135	0.81	0.19	150	1.00	0	137	1.00	0	152
Kwa Dzivo*	0.94	0.06	99	0.71	0.29	104	0.99	0.01	102	1.00	0	96
Puerto Rico*	0.99	0.01	692	1.00	0	673	0.58	0.42	108	1.00	0	143
A. mascarensis	0.98	0.02	118	1.00†	0	61	1.00	0	130	1.00	0	17
A. albopictus	0.78	0.22	91	1.00	0	76	1.00	0	99	0	1.00	14

The techniques of Ayala[5] were modified as follows. Esterase, no incubation in borate, 1.5 ml β-naphthyl acetate solution (1:1 water–acetone solvent) added; alkaline phosphatase, stain with no PVP, no NaCl; Coomassie blue, 60 mg Coomassie blue, 90 ml methanol, 18.3 ml glacial acetate, 91.7 ml water, after several hours washed with the same solution to clear background; malic enzyme, 0.056 M Tris-HCl buffer, PMS added only after 1 h incubation. Alleles are assigned positions relative to the distance travelled by the borate band, except for malic enzyme, for which the most frequent allele is assigned position 1.00. There are two esterase alleles at one locus[7]. For alkaline phosphatase, Coomassie blue and malic enzyme, band frequencies do not deviate from Hardy–Weinberg equilibrium assuming two Mendelian alleles at one locus.
*Sampled within one to two generations from field capture. Other samples were from laboratory colonies up to several years old.
†Gene frequency is for pupae only; older larvae have frequencies of 0.39, 0.61 (n = 28); half-grown larvae 0.95, 0.05 (n = 20).

breeds seasonally. Our data provide the first demonstration of electrophoretic differences between sympatric ecotypes.

The indoor and outdoor ecotypes used all came from an area (several square kilometres) around Rabai, near Mombasa, Kenya—the indoor samples from inside dwellings and the outdoor samples from feral habitats. The Kwa Dzivo sample came from eggs from tin cans nailed to trees in a village. A sample of *A. aegypti* from south-west Puerto Rico (Corozo, Maguayo, Sabana) in the Caribbean, *A. albopictus* from Singapore, and *A. mascarensis* from Mauritius in the Indian Ocean, were studied for comparison.

Crucial to the idea of sympatric polymorphism is the conspecificity of the two ecotypes. Both forms seem to be fully interfertile, with the colour pattern differences subject to continuous variation[4]. In our laboratory interbreeding occurred freely between the two ecotypes (the Mwamsabu indoor and the Bejumwa outdoor strains). Hybridisation experiments were carried out in the laboratory in cages with 20%, 50%, and 80% mixtures of the two ecotypes and 50% of each sex. Promiscuous coupling in *A. aegypti* precludes the use of coupling as a criterion of insemination. We therefore reared the offspring of each female and

Table 2 Gene frequencies of phosphoglucomutase alleles

	Locus A	Locus B						
Allele position	0.56	0.75	1.00	1.29	1.53	1.76	2.00	n
Indoor								
Rabai	0	0.05	0.60	0.35	0	0	0	54
Moyo	0	0.04	0.92	0.03	0	0	0	44
Mgandini*	0	0	0.82	0.13	0.06	0	0	95
Outdoor								
Rabai	0	0	1.00	0	0	0	0	46
Kombeni	0	0.01	0.99	0	0	0	0	44
Kombeni*	0	0.06	0.87	0.07	0	0	0	83
Kwa Dzivo*	0	0.04	0.86	0.10	0.01	0	0	91
Puerto Rico*	0	0	1.00	0	0	0	0	123
A. mascarensis	1.00	0	1.00	0	0	0	0	18
A. albopictus	0	0	0	0.04	0.19	0.58	0.19	13

The four alleles in *A. aegypti* were found by hybridising inbred lines to be at one locus[6], but band 0.56 of *A. mascarensis* is at a separate locus, and the alleles of *A. albopictus* may be at either or none of the previous loci. The most frequent allele (A1[5]) is designated position 1.00. Techniques are those of Ayala[5].
*Sampled within one to two generations from field capture. Other samples were from laboratory colonies up to several years old.

Table 3 Gene frequencies for leucine aminopeptidase

Sample	Locus A	Locus B		n
	0.59	0.76	0.81	
A. aegypti	1.00	0	0	1,675
A. mascarensis	1.00	0.98	0.02	61
A. albopictus	0	0	1.00	84

The techniques of Ayala[5] were modified as follows: incubation in 0.25 M borate; NaOH solution is 0.32 M not 0.2 M. Bands 0.76 and 0.81 are assumed to be two Mendelian alleles at one locus. Bands are assigned positions relative to the distance travelled by borate band. Fifteen bands were observed. In larvae band 0.70 stains heavily and predominates and band 0.66 seldom appears, whereas in pupae both bands, especially 0.66, usually appear and stain equally. Band 0.54 appears only in pupae in *A. albopictus*; it sometimes occurs in larvae of *A. mascarensis* and *A. aegypti*. Pupae of the latter two species sometimes have an additional band (0.36) next to band 0.40.

graded the abdominal scale pattern[4] to determine the ecotype of the male that inseminated each female. These experiments have so far not demonstrated any significant deviation from randomness in mating within and between ecotypes. Previous authors have always considered them to be one species[4,8]. We have demonstrated partial reproductive isolation between them; therefore, what we are calling two 'ecotypes' may actually be incipient species, partially isolated by habitat selection.

Persistence of the indoor ecotype in village huts seems to be the result of strong behavioural habitat selection for dwellings[9]. Such habitat selection may enable persistence of fairly discrete gene pools as shown by our data.

We will present elsewhere a mathematical model of the evolution of the indoor ecotype from the outdoor ecotype. We hypothesise that the indoor ecotype arose in sub-Saharan African areas with a dry season, when humans began to store water in pots in dwellings. During the dry season these pots are the only suitable habitat. We believe that habitat selection for the dwellings, and natural selection for different life history attributes and for preference for feeding on man arose during repeated dry seasons. The indoor ecotype has slower larval development, larger eggs, larger adults, and greater resistance to desiccation as adults. The indoor ecotype is preferentially attracted to human odour, the outdoor ecotype to guinea pig odour. Oxygen tension of water must be lower for eggs of the outdoor ecotype to hatch than for eggs of the indoor ecotype. Detailed evidence concerning the ecological differences between ecotypes will be published elsewhere.

JAMES A. SCOTT
G. A. H. McCLELLAND

Department of Entomology,
University of California,
Davis, California 95616

1 Ayala, F. J., *Evolutionary Biology* (in the press).
2 Johnson, G. B., *Science*, 184, 28 (1974).
3 McClelland, G. A. H., *Proc. Tall Timbers Conf. Anim. Control by Habitat Management, No. 5*, 27 (1974).
4 McClelland, G. A. H., *Trans. R. ent. Soc. Lond.*, 126, 239 (1974).
5 Ayala, F J., Powell, J. R., Tracey, M L., Mourao, C. A., and Perez-Salas, S, *Genetics*, 70, 113 (1972).
6 Bullini, L., Coluzzi, M., Bianchi Bullini, A. P., and Cancrini, G., *Parassitologia*, 15, 141 (1973).
7 Trebatoski, A. M., and Craig, G. B., Jr, *Biochem. Genet.*, 3, 383 (1969).
8 Mattingly, P. F., *Ann Trop. Med. Parasit.*, 51, 392 (1957).
9 Trpis, M., and Hausermann, W., *Bull. ent. Res.* (in the press)

Nitrogen fixation by *Rhizobium* cultured on a defined medium

IT has been widely believed that the legume root-nodule bacteria (*Rhizobium* spp.; rhizobia) fix N_2 only within the tissues of the host plant[1]. There have however been recent reports that a strain of cowpea rhizobia, 32H1, developed nitrogenase activity when grown in association with legume or non-legume plant cells[2,3]. The induction of nitrogenase was apparently due to a diffusible factor(s) secreted by the plant cells, since nitrogenase activity was detected when strain 32H1 was grown adjacent to, but not in contact with, tobacco cells[3]. Several plant metabolites, including sugars known to favour rhizobial growth[4,5] and citric acid cycle intermediates[6,7], were examined for their effect on this latter system and as possible direct inducers of nitrogenase activity in cultured rhizobia. This led to the formulation of a defined medium on which strain 32H1 fixed N_2 in the absence of plant cells.

This medium (CS7) contained (in mM) KH_2PO_4 (2.2), $CaCl_2.2H_2O$ (0.7), KCl (0.9), $MgSO_4.7H_2O$ (0.14), glutamine (2), myo-inositol (5.6), Na-succinate (25), L-arabinose (25) and (in μM) $MnSO_4.4H_2O$ (58), H_3BO_3 (82), $ZnSO_4.7H_2O$ (3.5), KI (6), $CuSO_4.5H_2O$ (0.8), $Na_2MoO_4.2H_2O$ (0.4), $CoCl_2.6H_2O$ (0.4), $FeSO_4.7H_2O$ (54), Na_2-EDTA (54), thiamine-HCl (15), nicotinic acid (41), pyridoxine-HCl (2.4), and 1% w/v agar (Difco Noble) at pH 5.9. In all experiments the bacteria were initially cultured on yeast-mannitol agar (YMA), suspended in sterile water (10^8–10^9 cells ml^{-1}) and spread as 1 cm^2 areas on CS7 medium. On other plates, similar areas of bacteria were grown 5 mm from uninoculated cell cultures of *Nicotiana tabacum* cv. Wisconsin 38. Cultures were incubated at 29 °C. Segments of agar bearing 1 cm^2 of dense bacterial growth were excised daily and assayed for nitrogenase activity by the acetylene reduction technique[8].

Nitrogenase activity was detected on day 4 and increased linearly during the following 4 d (Fig. 1). The rates of C_2H_2 reduction were similar for bacteria cultured in the presence or absence of plant cells. When arabinose (Fig. 1), succinate, or glutamine was omitted from the CS7 medium, nitrogenase activity was low or undetectable. Fumarate was, however, found to be a suitable replacement for succinate and galactose could replace arabinose in the basic medium. The bacteria also showed nitrogenase activity when $(NH_4)_2SO_4$ (1 mM) replaced glutamine in the medium, but the level of activity was less. The reduction in the glutamine concentration from that used in previous studies of plant cell–rhizobia associations[3,7,10] followed experiments which showed that 2–10 mM was the optimum concentration range for nitrogenase activity in the tobacco-strain 32H1 association.

The optimal P_{O_2} for C_2H_2 reduction was found to be 0.15–0.20 atm (2.5 and 1.9 nmol C_2H_4 per segment per h

Fig. 1 Time course of acetylene reduction by strain 32H1 cultured on CS7 medium. Agar segments (1 cm^2) containing bacteria (about 2 mg bacterial protein[9] per segment at 8 d) were sealed in vials with serum stoppers, evacuated, and filled with a gas mixture containing 20% O_2: 10% C_2H_2: 70% argon, incubated at 30 °C for 2–3 h and assayed for C_2H_2 reduction by gas chromatography. Activity on CS7 with ● and without ○ arabinose; activity on CS7 plus tobacco cells (see text) with ▲ and without △ arabinose.

Table 1 Strains* of different *Rhizobium* species assayed for C_2H_2 reduction when cultured on CS7 medium

Rhizobium spp.	Nitrogenase positive†	Nitrogenase negative
Rhizobium sp. (Cowpea group)	CB756, CC751, CC1066, 41F2, 61B9, 127N1, 127N2	CC402, CC430, NU197, 32Z3, 29C2, 8B4, 41A1
R. japonicum	61A76, CB1809, CC705, CC715, CC717, CC713, CC716, CC723	129, CC714, CC718, CC720, CC721 WU274, WU425, CC603, CC624
R. lupini	—	CC8, SU47, SU388, SU574
R. meliloti	—	
R. trifolii	—	TA1, WU95, CC2238c, WA67, 2009
R. leguminosarum	—	TA101, NA505

*The strains, all of which nodulate "Siratro" (including *R. japonicum*) or their homologous hosts, were provided by Dr J. C. Burton, Nitragin Co., Milwaukee, USA, Dr D. A. Phillips, Indiana State University, Terre Haute, USA, Dr J. Dobereiner, IPEACS, Km 47, Campo Grande, Brazil, and J. Brockwell, Division of Plant Industry, CSIRO, Canberra, and we are grateful for their cooperation. Details of strain isolation and source will be provided on request to the authors.
†All positive strains produced >0.5 nmol C_2H_4 per culture per h, with the exception of CC705 (0.2 nmol C_2H_4 per h).

respectively). In assays without O_2 added to the gas mixture (10% C_2H_2, 90% argon), or with $P_{O_2} = 0.4$ atm, the activity was 0.1 nmol C_2H_4 per segment per h.

Strain 32H1 was cultured on CS7 medium for 7 d and exposed to an atmosphere of 20% O_2 and 80% N_2, containing 80 atoms % ^{15}N, for 24 h. The bacteria were washed from the agar and ^{15}N enrichment of the total N of the samples determined[8]. The highly significant values of 0.948 and 0.643 atom % excess for two samples, compared with zero values for control bacteria incubated in air, indicated that the cultures fixed N_2.

In previous experiments, 32H1 was shown to be a pure culture of rhizobia[2,3]. We have subsequently examined the following clones of strain 32H1 (1) 15 single colonies isolated from YMA cultures[3], (2) 12 single colonies isolated from nodules formed following inoculation of cowpea plants with strain 32H1[3] (3 isolates from 4 nodules) and (3) 12 single colonies isolated from 32H1 segments on CS7, replicates of which were active in reducing C_2H_2 (Fig. 1). All of these isolates reduced C_2H_2 when cultured on CS7, and also nodulated the tropical legume "Siratro" (*Macroptilium atropurpureum*) grown from surface-sterilised seed on sterile agar slopes in cotton-plugged tubes. No natural C_2H_4 production by strain 32H1 cultured on CS7 was detected, nor did this strain reduce C_2H_2 when grown on YMA medium, or on media[3] used previously. Ten single colonies of 32H1 growing on CS7 failed to grow when transferred to nutrient agar (Difco). We therefore concluded that the cultures which fixed N_2 were authentic, pure cultures of cowpea rhizobia.

In addition to strain 32H1, 42 strains from 5 *Rhizobium* species and the cowpea group (*Rhizobium* spp.) were tested for nitrogenase activity on CS7 (Table 1). Of the 6 active strains from the cowpea group, strain CB756, and all 12 single colony isolates from nodules on CB756-inoculated cowpea plants, showed a level of activity comparable to that found with strain 32H1. Three of the strains (61A76, 29C2, 32Z3) tested here were reported previously to form an N_2-fixing association with soybean cells[11-13]; of these, only *R. japonicum* 61A76 showed nitrogenase activity on CS7.

These results show that some rhizobia strains can exhibit nitrogenase activity when cultured on a defined medium in the absence of plant cells. This provides direct proof that these rhizobia possess the genetic information for the biosynthesis of the nitrogenase enzyme, thus confirming earlier suggestions of an indirect nature based on genetic[14] and biochemical[15,16] evidence and results of tissue culture experiments[2,3]. Significant levels of nitrogenase activity seem to depend on the provision of preferred carbon (for example arabinose, galactose) and nitrogen (for example glutamine, $(NH_4)_2SO_4$) sources for bacterial growth and a citric acid cycle intermediate (for example succinate, fumarate). The glutamine-synthesising enzymes are important in the regulation of nitrogenase synthesis and ammonia assimilation in *Klebsiella*[17,18], and although these enzymes are found in nodule bacteroids[19,20], our current results do not permit conclusions regarding a regulatory function for these enzymes in rhizobia. So far, attempts to demonstrate workable levels of nitrogenase activity by strain 32H1 in CS7 liquid culture have been unsuccessful. We suggest

that N_2 fixation by rhizobia may depend on the development of favourable conditions within the mass of bacterial growth.

We thank Mr G. L. Turner for the ^{15}N analyses and J. Adamson, M. Ashby, Y. Hort, and L. McCurley for their technical assistance. Dr J. J. Child is currently on leave from the NRC Prairie Regional Laboratory, Saskatoon, Canada.

J. D. PAGAN
J. J. CHILD
W. R. SCOWCROFT
A. H. GIBSON

Division of Plant Industry,
CSIRO,
PO Box 1600,
Canberra City, ACT 2601,
Australia

1 Quispel, A, in *The Biology of Nitrogen Fixation* (edit. by Quispel, A.), 719 (North-Holland, Amsterdam, 1974).
2 Child, J. J., *Nature*, 253, 350–351 (1975).
3 Scowcroft, W. R, and Gibson, A. H., *Nature*, 253, 351–352 (1975).
4 Fred, E. B, Baldwin, I. L., and McCoy, E, *Root Nodule Bacteria and Leguminous Plants*, 86 (University of Wisconsin Press, Madison, 1932).
5 Graham, P H, *Antonie van Leeuwenhoek*, 30, 68–72 (1964)
6 Child, J. J, and LaRue, T. A, in *Proc. Int. Sym. N₂ Fixation, 1974* (Pullman, Washington, in the press)
7 Phillips, D. A., *Pl Physiol., Lancaster*, 54, 654–655 (1974).
8 Bergersen, F. J, *Aust. J. biol Sci.*, 23. 1015–1026 (1970).
9 Lowry, O. H, Rosebrough, N. J, Farr, A. L., and Randall, R J., *J. biol. Chem.*, 193, 265–275 (1951).
10 Phillips, D. A., in *Proc. Int. Symp N₂ Fixation, 1974* (Pullman, Washington, in the press)
11 Holsten, R. D, Burns, R. C., Hardy, R. W. F, and Hebert, R. R., *Nature*, 232, 173–176 (1971).
12 Child, J J., and LaRue, T. A., *Pl. Physiol., Lancaster*, 53, 88–90 (1974).
13 Phillips, D. A, *Pl. Physiol., Lancaster*, 53, 67–72 (1974)
14 Dunican, L. K, and Tierney, A. B., *Biochem. biophys. Res. Commun*, 57, 62–73 (1974).
15 Phillips, D. A., Howard, R. L., and Evans, H. J., *Physiol. Pl.*, 28, 248–253 (1973).
16 Bishop, P E., Evans, H. J, Daniel, R. M, and Hampton, R. O., *Biochem. biophys Acta*, 381, 248–256 (1975).
17 Streicher, S. L, Shanmugam, K. T., Ausubel, F, Morandi, C., and Goldberg, R B, *J. Bact*, 120, 815–821 (1974).
18 Tubb, R S., *Nature*, 251, 481–485 (1974).
19 Dunn, S D, and Klucas, R V., *Can. J. Microbiol*, 19, 1493–1499 (1973)
20 Brown, C. M, and Dilworth, M. J., *J. gen. Microbiol.*, 86, 39–48 (1975).

Nitrogenase activity in rhizobia in absence of plant host

RHIZOBIA can infect legume roots and fix dinitrogen in the root nodules. Repeated attempts have failed to demonstrate fixation by rhizobia on defined medium or in plant extracts. *Rhizobium japonicum* grown anaerobically with nitrate produces a haemoprotein pattern similar to that of N_2-fixing bacteroids[1] and synthesises the Mo–Fe protein of nitrogenase[2], but it has seemed that infection of legume cells was a prerequisite for the expression of nitrogenase. It has been shown recently[3,4], however, that cowpea *Rhizobium* 32H1 could fix dinitrogen when grown close to, but not in contact with, callus cultures of legumes or non-legumes. This supported other evidence[5,6] that it was the rhizobia which contained the genetic information for nitrogenase, and showed that its expression was promoted by diffusable factors from plant cells.

To characterise these factors we tested callus cultures of

several plant species, and found carrot and rice most effective. Extracts of carrot cells, or carrot root, when added to LNB5 medium[7] permitted the expression of nitrogenase (C_2H_2) activity by cowpea *Rhizobium* 32H1 grown in the absence of plant cells. We also screened carbohydrates for their use in promoting the callus–*Rhizobium* association[7], and found xylose, arabinose and galactose particularly stimulating. Subsequently it was found that when plant callus was omitted, one or the other of these sugars plus sucrose sufficed to induce nitrogenase activity. This communication describes nitrogenase activity in rhizobia growing alone on defined medium.

Rhizobial strains were maintained on a medium containing K_2HPO_4 0.5 g; $MgSO_4.7H_2O$ 0.2 g; $NaCl$ 0.1 g; yeast extract 0.5 g; $CaCO_3$ 0.5 g; mannitol 10 g; per litre of water. The rhizobia produced effective nodules on their appropriate hosts. Neither growth nor acetylene reduction were observed when the bacteria were subcultured on nitrogen-free media[3].

The LNB5 medium[7] contained KNO_3 1,000 mg; $(NH_4)_2SO_4$ 50 mg; $MgSO_4.7H_2O$ 250 mg; $NaH_2PO_4.H_2O$ 150 mg; $CaCl_2.2H_2O$ 150 mg; Fe (as Sequestrene 330) 28 mg; $MnSO_4$ 10 mg; H_3BO_3 3 mg; $ZnSO_4.7H_2O$ 2 mg; $Na_2MoO_4.2H_2O$ 0.25 mg; $CuSO_4$ 0.025 mg; $CoCl_2.6H_2O$ 0.025 mg; KI 0.78 mg; myo-inositol 100 mg; thiamine. HCl 10 mg; nicotinic acid 1 mg; pyridoxine. HCl 1 mg; sucrose 30 g; agar 12 g l^{-1}. The pH was adjusted to 5.5.

Cowpea *Rhizobium* 32H1 reduced acetylene when grown on a defined modified LNB5 medium containing galactose, arabinose or xylose together with sucrose (Table 1). For reasons not clear to us, nitrogenase activity is absent if only one carbon source is available.

A source of fixed nitrogen is necessary for dinitrogen fixation by *Rhizobium* under our *in vitro* conditions. In the case of *R. japonicum* 61A76, there is no dinitrogen fixation when fixed nitrogen is not supplied (Table 2). Fixation occurs in the presence of relatively high levels of ammonia, and is not significantly changed in the presence of L-methionine sulphone, which can interfere with NH_3 repression of nitrogenase. In these respects, *R. japonicum* differs from some other N_2-fixing organisms such as *Azotobacter vinelandii* and *Klebsiella pneumoniae*[9]. Ammonia can be replaced as N source by 0.002 M glutamine, glutamic acid and aspartic acid. Alanine and leucine are less effective and only slight dinitrogen fixation is obtained with valine or citrulline.

R. leguminosarum TA101 fixes nitrogen on a defined nutrient containing xylose (Table 3). There is scant growth and no fixation in the absence of nitrate. Ammonia apparently neither inhibits nitrogenase nor permits its expression in the absence of nitrate.

Dinitrogen fixation in the presence of fixed nitrogen may seem remarkable, for it is often assumed that nitrogenase is always repressed by fixed nitrogen. Some strains of *Azotobacter vinelandii* will fix dinitrogen in the presence of nitrate, however,

Table 2 Effect of ammonia and methionine sulphone on nitrogenase activity of *R. japonicum* 61A76

$(NH_4)_2SO_4$ concentration	Nitrogenase specific activity nmol C_2H_4 per h per mg protein
0.001 M	1.85
0.001 M+MSO	1.89
0.01 M	1.39
0.01 M+MSO	1.16
0.1 M	0
0.1 M+MSO	0
0+MSO	0
0	0

R. japonicum 61A76 was added to the surface of 10 ml solid medium slant in a 30-ml test tube. The nutrient was LNB5 except that the carbon source was arabinose (10 g l^{-1}) and sucrose (10 g l^{-1}) and nitrate was omitted. Methionine sulphone (MSO) was added at 3 mg ml^{-1}. After 9 d, nitrogenase activity and protein content were measured. Each figure is the average of three samples.

and may use both sources simultaneously[10], and in chemostat cultures of *A. chroococcum*, stable cultures are obtainable in which nitrogenase is only partially repressed by ammonia[11]. There is no reason to believe that the controls on nitrogenase in rhizobia are exactly the same as those in *Azotobacter* or *Clostridium*, and the symbiotic bacteria may require higher levels of fixed nitrogen to repress the enzyme. It has already been established elsewhere that *R. japonicum* can synthesise the Mo–Fe protein in the presence of ammonia and nitrate[2].

We only observe acetylene reduction in strains which produce a copious slime. It is probable that this serves to protect the nitrogenase from oxygen[12]. The bacteria near the surface of the colony may lower the oxygen tension whereas those below produce nitrogenase. In this case, the specific activities underestimate the activity of the nitrogen-fixing rhizobia.

Table 3 Nitrogenase activity in *R. leguminosarum* TA101

Nitrogen source (mg l^{-1}) KNO_3	$(NH_4)_2SO_4$	nmol C_2H_4 per h per mg protein
1,000	50	0.91
—	50	0
1,000	—	0.97
—	—	0

R. leguminosarum TA101 was added to the surface of 10 ml solid medium slanted in a 30-ml test tube. The nutrient was LNB5 except that the carbon source was xylose (10 g l^{-1}) and sucrose (20 g l^{-1}). Nitrogenase activity and protein were determined after 14 d.

The significance of our results rests on the assumption that our rhizobial cultures are uncontaminated by nitrogen-fixing bacteria. Absence of *Klebsiella* was demonstrated by lack of growth, acid or gas formation in anaerobic semi-solid peptone media containing mannitol, sucrose, glucose or xylose[8]. Lack of growth on standard nitrogen-free media[3] and fixation at pH 5.5 also make it unlikely that our cultures are contaminated by *Azotobacter*. Fixation is not observed if test cultures are maintained under dinitrogen, and this argues against the presence of *Clostridium* or *Bacillus*. We have not detected acetylene reduction in any experiment in which sucrose, glucose, fructose or mannitol was provided as sole carbon source. Fixation was promoted by xylose or arabinose, however. Because pentoses are preferred carbon sources by rhizobia, it is certain that the acetylene reduction activity we observe is due to the non-symbiotic nitrogenase activity by rhizobia.

W. G. W. KURZ
T. A. LARUE

National Research Council of Canada,
Prairie Regional Laboratory,
Saskatoon, Saskatchewan S7N 0W9, Canada

Table 1 Effect of carbon source on nitrogenase activity of cowpea *Rhizobium* 32H1

Carbon source (g l^{-1})		Nitrogenase specific activity nmol C_2H_4 per h per mg protein
Sucrose	20	0
Arabinose	20	0
Sucrose +mannitol	10 10	0
Sucrose +xylose	10 10	0.47
Sucrose +arabinose	10 10	0.82
Sucrose +galactose	10 10	0.35

Cowpea *Rhizobium* 32H1 was added to the surface of 10 ml solid medium slant in a 30-ml test tube. The nutrient was the LNB5 medium except that the carbon source was varied as shown in the table. After 6 d, nitrogenase activity and protein content were measured. Each figure is the average of four samples.

1 Daniel, R. M, and Appleby, C. A., *Biochim biophys. Acta*, **275**, 347–354 (1972).

2 Bishop, P. E., Evans, H. J., Daniel, R. M., and Hampton, R. O., *Biochim.*
    biophys. Acta, 381, 248–256 (1975).
3 Child, J. J., *Nature*, 253, 350–351 (1975)
4 Scowcroft, W. R., and Gibson, A. H., *Nature*, 253, 351–352 (1975).
5 Dunican, L. K., and Tierney, A. B., *Biochem. biophys. Res. Commun.*, 57, 62–72
    (1973)
6 Phillips, D. A., Howard, R. L., and Evans, H. J, *Physiol. Pl.*, 28, 248–253 (1973).
7 Child, J. J., and LaRue, T. A., *Pl Physiol.*, *Lancaster*, 53, 88–90 (1974).
8 Hugh, R., and Leifson, E., *J. Bact.*, 6, 24–26 (1953).
9 Gordon, J. K., and Brill, W. J., *Biochem. biophys. Res. Commun.*, 59, 967–971
    (1974).
10 Wilson, P. W., in *Encyclopaedia of Plant Physiology*, VIII, (edit. by Ruhland, W.),
    (Springer, Berlin, 1958).
11 Hill, S., Drozd, J. W., and Postgate, J. R., *J. appl. Chem. Biotechnol.*, 22, 541–552
    (1972).
12 Postgate, J. R., *The Chemistry and Biochemistry of Nitrogen Fixation* (Plenum,
    London, 1971).

Acetylene reduction by *Rhizobium* in pure culture

ASSOCIATIONS between legume callus cultures and *Rhizobium* have been shown to fix nitrogen[1-6]; we have found that at least one strain of *Rhizobium* will do so when cultured alone on certain media.

The strains of *Rhizobium* used were: 32H1, a cowpea strain (Dr J. Burton, Nitragin Co., Milwaukee, Wisconsin); NGR46, another cowpea strain (Dr M. J. Trinick, CSIRO Wembley, Western Australia); and MU1, an isolate from nodules of *Trema cannabina*, (Mr D. R. Coventry, University of Western Australia). MU1 is a strain able to nodulate siratro (*Macroptilium atropurpureum*) and cowpea (*Vigna unguiculata* ssp. *unguiculata*). *Rhizobium* cultures were maintained on a yeast extract–mannitol–potato extract medium[7].

Confirmation that the initial culture of 32H1 was a *Rhizobium* was obtained by testing it for ability to nodulate cowpea. One series of four pots, each containing five plants, was inoculated with 32H1, another series with NGR46, while a third was left uninoculated as a control. Ten days after the seed inoculation, the appropriate pots were each treated with 100 ml of the test *Rhizobium* suspended in sterile water to give about 10^9 bacteria ml^{-1}. The controls remained unnodulated, while NGR46 nodulated all plants, as did 32H1. A strain of 32H1 was recovered from the nodules and used in further experiments (it is referred to as the first reisolate).

Cultures from eight individual colonies of the first reisolate were then used to each inoculate pots containing five cowpea plants. Suitable uninoculated controls were again included, and did not form nodules. Twenty-two clones of 32H1 were recovered, at least one from each pot, and these clones are referred to as the second reisolates.

A rabbit antiserum prepared against the first reisolate of 32H1 (ref. 8) showed that the initial strain, the first reisolate and the second reisolates were serologically indistinguishable.

The *Rhizobium* plant symbioses set up[2,3] used soybean (*Glycine max*) and snake bean (*Vigna unguiculata* ssp. *sesquipedalis*) suspension cell cultures and rhizobial strains 32H1, NGR46 and MU1. *Rhizobium* cultures for inoculation of control bottles were derived from cultures grown on maintenance medium (broth or solid) and were adjusted to contain 1.0×10^7–9.0×10^7 bacteria ml^{-1} in sterile water. The flat surface of 15 ml of callus medium in a 30 ml McCartney bottle was inoculated with 0.1 ml of rhizobial suspension. The callus culture medium was SCN (ref. 2) with or without 10 mM glutamine and 40 mM succinate[3]. The succinate was added as succinic acid, which lowered the pH of the callus medium to 2.5–3.0; the pH was then returned to 6.0 with KOH before adding the agar. Cultures were grown in diffuse light at 25 °C.

After 14 d, the aluminium caps on the cultures were removed and replaced with a black rubber seal, and an aluminium cap with a hole. Endogenous ethylene was read 24 h later on a Pye-Unicam gas chromatograph fitted with a 1.4 m column of Porapak T and operated at 100 °C. The cultures were then flushed with sterile air in a laminar flow cabinet before the caps were replaced and 0.75 ml of acetylene injected. After 24 h, ethylene was again measured, and acetylene-dependent

ethylene production calculated by subtracting the endogenous production.

Plant calluses with or without rhizobia showed an endogenous ethylene production of 0.08 nmol or less per culture bottle. Rhizobia on callus media had an endogenous ethylene production usually undetectable and never greater than 0.01 nmol.

Soybean callus cultures inoculated with strain 32H1 reduced acetylene at rates comparable to those reported by Phillips[2,3], but inoculated callus cultures of snake bean did not do so (Table 1). Strain 32H1 also performs poorly with calluses

Table 1   Acetylene-dependent ethylene production by various rhizobial strains, callus cultures, and associations between them

Rhizobium strain	Plant callus	Mean ethylene production per culture (nmol)	Standard error
None	None	0.08	± 0.02
MU1	None	0.08	± 0.02
NGR46	None	0.10	± 0.02
32H1	None	37.89	± 6.93
None	Snake bean	1.24	± 0.53
None	Soybean	0.03	± 0.02
NGR46	Snake bean	0.09	± 0.06
32H1	Snake bean	1.41	± 0.62
32H1	Soybean	68.39	±24.98

All cultures on SCN medium supplemented with 10 mM glutamine and 40 mM succinate.

of *Vigna unguiculata*[5], and varies in performance with different soybean cultivars[2]. Internal variability was often high, as reported by others[2,4].

Neither the plant calluses alone nor bacterial strains NGR46 and MU1 showed acetylene reduction. Cultures of 32H1, however, reduced acetylene actively on the SCN medium supplemented with glutamine and succinate.

The first reisolate also reduced acetylene on SCN medium supplemented with glutamine and succinate; neither it nor the initial culture did so on SCN alone, or on SCN supplemented only with glutamine. Twenty-two of the second reisolate clones of 32H1 reduced acetylene on SCN supplemented with glutamine and succinate, but not on SCN alone (Table 2). Since 32H1 associations with callus cultures incorporate $^{15}N_2$ (ref. 5), we assume that acetylene-dependent ethylene production can be attributed to nitrogenase activity.

Contamination by nitrogen-fixing bacteria is an unlikely explanation for our results. It seems improbable that the initial culture and the isolate from the first nodule passage should both have contained a nitrogen-fixing contaminant, and even less probable that eight individual colonies from an apparently pure culture could undergo a second nodule passage and again be reisolated with a nitrogen-fixing contaminant in each of the twenty-two second reisolates. We conclude, therefore, that 32H1 is a *Rhizobium* strain capable of acetylene reduction in these conditions.

Table   2 Acetylene-dependent ethylene production by *Rhizobium* strain 32H1 after second nodule passage

First isolate colony no.	Mean ethylene production (nmol) on		Standard error
	SCN	SCN+glutamine+succinate	
1	0	20.53	± 5.33
2	0	25.97	±17.87
3	0	20.34	± 4.80
4	0	18.44	± 7.93
5	0	55.10	±15.02
6	0	54.68	±13.47
7	0	20.95	± 7.64
8	0	34.71	± 6.68
Mean (77 observations)	0	34.04	± 4.67

The medium used was SCN, or SCN supplemented with 10 mM glutamine and 40 mM succinate.

Table 3 Acetylene-dependent ethylene production by the first reisolate of *Rhizobium* strain 32H1 on SCN medium with various supplements

Additions	Mean ethylene production (nmol)	Standard error
None	0.00	± 0.00
10 mM glutamine	3.17	± 1.42
40 mM succinic acid	0.00	± 0.00
10 mM glutamine+40 mM succinic acid	31.69	± 6.64
10 mM glutamic acid	21.64	± 5.45
10 mM glutamic acid+40 mM succinic acid	40.59	±10.58
40 mM Na succinate	0.00	± 0.00
10 mM glutamine+40 mM Na succinate	11.16	± 2.39
10 mM asparagine	0.79	± 0.04
10 mM asparagine+40 mM succinic acid	25.79	± 6.74

All amino acids are the L-isomer.

We examined the effects of various media supplements on acetylene reduction by 32H1 (Table 3). Glutamine and asparagine were equivalent in the presence of succinic acid, but glutamine in the presence of sodium succinate was significantly inferior. The acidity of succinic acid was apparently important, but only where an amide nitrogen compound was used. Since neither succinic acid nor sodium succinate alone was effective, an available nitrogen source was apparently necessary. The significant increase in acetylene reduction when succinic acid was added with glutamate (which is acid stable) suggests that an available carbon source was also needed. Since slow-growing rhizobia do not usually grow on disaccharides[9], the sucrose in the callus medium would be ineffective.

Given that glutamine and asparagine are not available for growth of 32H1, and that relatively slight hydrolysis of them to available glutamate, aspartate or ammonia occurs when they are treated with succinic acid, it seems probable that the responses can be explained by growth requiring available sources of carbon and nitrogen.

Acetylene reduction occurs with 32H1 in association with many types of callus, including non-legumes[2,3,5,6], and when callus and rhizobia are not in physical contact[5], suggesting that the calluses provide substances for growth or nitrogenase production. Snake bean callus seemed to prevent nitrogenase production, since the medium contained the necessary supplements for 32H1 alone (Table 1). This conclusion must remain tentative, since rhizobial numbers in the callus and agar surface systems are not comparable.

The finding that strain 32H1 reduces acetylene on laboratory media directly establishes that genes for nitrogenase are present in *Rhizobium*, as suggested by other workers[10,11] and renders invalid the theory[12] that part of the nitrogenase in legume nodules might be specified by plant DNA.

We thank Mrs J. Ryan for technical assistance and Dr D. L. Chatel of the Western Australian Department of Agriculture for serologically typing the various cultures and the Rural Credits Development Fund and the Australian Research Grants Committee for financial support.

J. A. McComb*
J. Elliott*
M. J. Dilworth*

*Departments of Agronomy and Soil Science and Plant Nutrition,
University of Western Australia,
Nedlands, Western Australia 6009*

1 Holsten, R. D., Burns, R. C., Hardy, R. W. F., and Hebert, R. R., *Nature*, 232, 173–176 (1971).
2 Phillips, D. A., *Pl. Physiol., Lancaster*, 53, 67–72 (1974).
3 Phillips, D. A., in *Proc. Int. Symp. N₂ Fixation, 1974* (Pullman, Washington, in the press)
4 Child, J. J., and LaRue, T A., *Pl. Physiol., Lancaster*, 53, 88–90 (1974)
5 Scowcroft, W R., and Gibson, A. H., *Nature*, 253, 351–352 (1975).
6 Child, J. J., *Nature*, 253, 350–351 (1975)
7 Godfrey, C. A., and Dilworth, M. J., *J. gen. Microbiol.*, 69, 385–390 (1971).
8 Vincent, J. M. *A Manual for the Practical Study of Root Nodule Bacteria* (Blackwell, Oxford, 1970).
9 Graham, P. H , *Antonie van Leeuwenhoek*, 30, 68–72 (1964).
10 Phillips, D. A., Howard, R. L., and Evans, H. J., *Physiol. Pl.*, 28, 248–253 (1973).
11 Dunican, L K., and Tierney, A. B., *Biochem. biophys Res. Commun.*, 57, 62–72 (1974).
12 Dilworth, M. J., and Parker, C. A., *J. theor. Biol*, 25, 208–218 (1969).

High frequencies of haploid production in wheat (*Triticum aestivum*) by chromosome elimination

The production of haploid plants from hybrids, followed by chromosome doubling, provides the plant breeder with a means of accelerating the development of desirable true breeding lines. This could be advantageous in breeding programmes particularly among some of the cereals where many generations, taking many years, may be required before an acceptable pure line variety can be obtained.

A major limitation to haploid breeding is the lack of techniques for obtaining haploids in sufficiently large numbers. In cultivated barley (*Hordeum vulgare* $2n=14$) the discovery[1] of a high frequency of "vulgare" haploids in hybridisations with *Hordeum bulbosum* ($2n=14$) by means of chromosome elimination, combined with recent improvements in cultural techniques[2-4], promises to overcome the limitation in this cereal.

So far the possibility that chromosome elimination giving rise to haploids may be exploited in other crop plants has not been investigated. To determine if elimination can occur in hexaploid wheat, *Triticum aestivum* ($2n=6x=42$), crosses were made between the wheat variety Chinese Spring and both diploid and tetraploid *H. bulbosum* as pollen parents. Seed set was obtained in both crosses (Table 1) although

Table 1 Seed set induced from intergeneric crosses between *T. aestivum* var. Chinese Spring and *H. bulbosum*

Male parent	No. florets pollinated	No. of seeds set	Seeds set (%)
2x *H. bulbosum*	622	85	13.7
4x *H. bulbosum*	435	188	43.2

the frequency was much higher using tetraploid *H. bulbosum*. The initial development of the seeds was vigorous but after 14–18 d the seeds showed signs of abortion, the endosperm having degenerated, making embryo culture necessary. The embryos were dissected out in a sterile cabinet, transferred to orchid agar (27–29 g l⁻¹) and placed in the dark at 20 °C until they germinated.

Of the 70 plants obtained (Table 2), 57 were examined cytologically in root tips stained by the Feulgen procedure and, whether produced from crosses with diploid or tetraploid *H. bulbosum*, all were found to have 21 chromosomes indistinguishable karyotypically from the haploid complement of wheat (Fig. 1). Nor did any of the plants as seedlings, or the ten plants which have reached maturity so far, exhibit any of the morphological features of *H. bulbosum*.

The frequency with which haploids were obtained when

Table 2 Plants obtained from intergeneric crosses between *T. aestivum* var. Chinese Spring and *H. bulbosum* using embryo culture

Male parent	No. of embryos cultured	No. of plants produced	Embryos giving plants (%)
2x *H bulbosum*	39	11	28.2
4x *H. bulbosum*	110	59	53.6
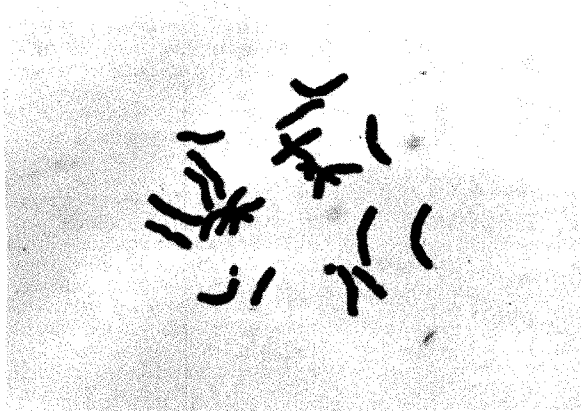
Fig. 1 Somatic chromosomes of a haploid wheat plant (2n = 21) obtained by crossing *Triticum aestivum* var. Chinese Spring with diploid *Hordeum bulbosum*. The satellited chromosomes 1B and 6B of wheat are clearly visible. ×750

using tetraploid *H. bulbosum* (13.6% of florets pollinated) is similar to the frequencies being achieved in haploid barley production. The mechanism of production of these haploids also appears to be the same as that for haploid barley from crosses of *H. vulgare* with *H. bulbosum* in which elimination of the *H. bulbosum* chromosomes takes place following fertilisation. In developing seeds fixed 1–6 d after pollination with tetraploid *H. bulbosum*, chromosome counts of 35 at zygotic metaphase indicated that fertilisation was normal. At a later stage, however, the presence of micronuclei in both embryo and endosperm tissues as well as lagging chromosomes and bridges in endosperm divisions (Fig. 2) showed the subsequent elimination of chromosomes.

Fig. 2 Chromosome elimination in a dividing endosperm cell of a developing grain obtained by crossing *Triticum aestivum* var. Chinese Spring with tetraploid *Hordeum bulbosum*. ×700
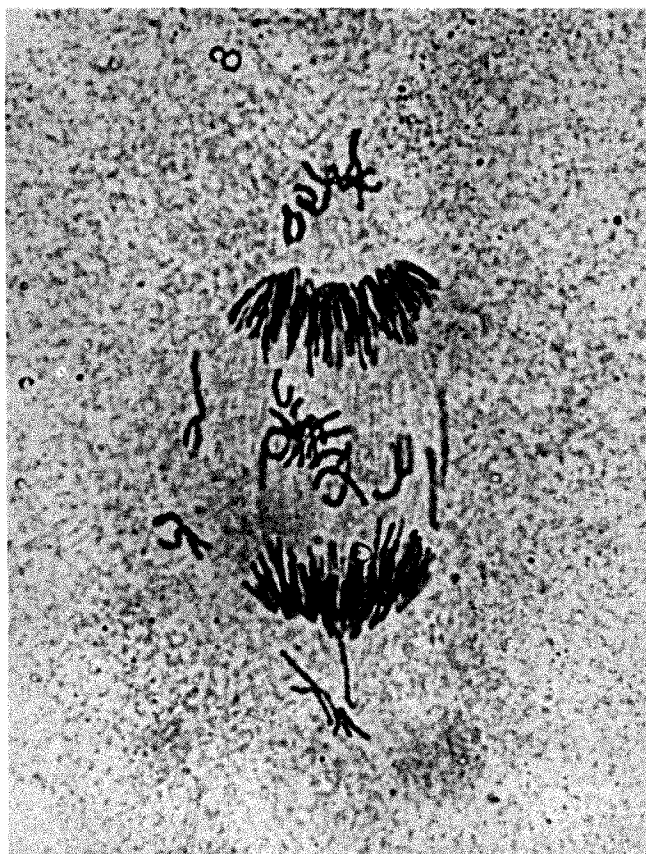
Investigations are now being carried out to determine whether *H. bulbosum* can be crossed readily with other wheat varieties. These initial results, however, demonstrate the potential of producing haploid wheats by means of chromosome elimination following hybridisation with *H. bulbosum*.

I. R. BARCLAY

Plant Breeding Institute,
Cambridge, UK

1 Kasha, K. J., and Kao, K. N., *Nature*, **225**, 874–876 (1970).
2 Islam, R., and Sparrow, D. H. B., *Barley Newslett.*, **17**, 40–42 (1974).
3 Subrahmanyam, N. C., and Kasha, K. J., *Barley Gen. Newslett.*, **3**, 62–65 (1973).
4 Kasha, K. J., in *Haploids in Higher Plants, Advances and Potential*, 67–87 (University of Guelph, 1974).

Visual spatial summation in two classes of geniculate cells

VISUAL perception can be viewed as a transformation and a distortion of the spatial patterns which exist in the outside world. The transformation of external objects into neural images is accomplished by the summation of excitatory and inhibitory influences over the visual field of each neurone. Distortion occurs when the neural response is not simply proportional to the sum of excitation and inhibition because of nonlinear mechanisms in spatial summation. If the responses of all visual cells were distorted by such nonlinear summation, fine visual discriminations like those necessary for reading *Nature* would be impossible. Nonlinear mechanisms do however, seem to be important in one class of visual neurone, perhaps for signalling change or motion in the external world. We have attempted to understand the importance of the different kinds of spatial summation by studying single cells in the cat visual system.

There are several classes of ganglion cells in the cat retina. Two main groups of these cells send axons to the dorsal lateral geniculate nucleus (LGN). These were originally classified and non-committally named X and Y cells by Enroth-Cugell and Robson[1]. They found that the main distinction between X and Y ganglion cells is the degree of linearity of spatial summation. A stationary spatial grating, amplitude-modulated in time can be located over the receptive field of an X cell so as to produce no modulation of the impulse rate of the cell. This position of the grating is called the null position. For even a slight relocation of the pattern away from the null position, modulation of the pattern produces modulation of the impulse rate. For Y ganglion cells no null position exists. Signals generated by a change in illumination over one region of the field of a Y cell can never be completely cancelled by signals due to an equal but opposite change in illumination in another region and therefore the Y cells must have some nonlinearity preceding, or at the level of, spatial summation. We have extended this work on the linearity of spatial summation to cat LGN cells. Geniculate cells can also be classified as X or Y on the basis of the linearity or nonlinearity of spatial summation. So X and Y cells are segregated at least as far as the input to the visual cortex. Other workers have used different criteria for grouping retinal ganglion and lateral geniculate cells[2-4]. These criteria include: axonal conduction velocity, preferential target speed, pattern of response to a drifting grating and response dynamics. We have not investigated the first two of these, but have measured the last two. Elevation of the mean impulse rate in response to a drifting grating is invariably present in Y cells, and we think this characteristic is caused by the same nonlinearity in spatial summation which prevents the cell from having a null position. There is, however, no direct relationship between spatial summation and response dynamics. There are some "transient" X cells and "sustained" Y cells. Because the spatial summation properties of X and Y cells are so distinct, there must be func-
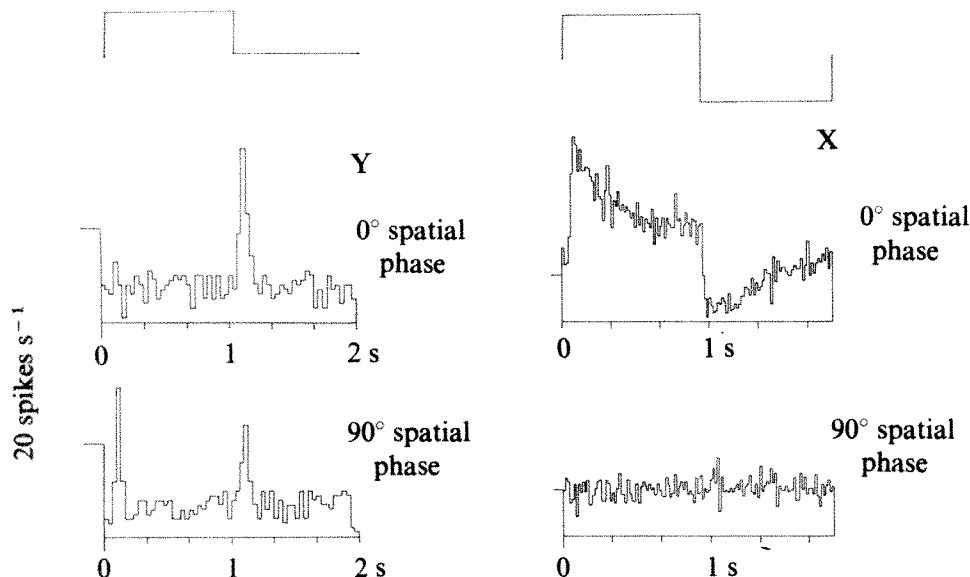
Fig. 1 Null positions in geniculate cells. The right hand column contains two responses (PST histograms of 30 sweeps) of an X geniculate cell to an alternating phase spatial sine grating, spatial frequency 1.8 cycles per degree, temporal frequency 0.54 Hz. The upper response was elicited by the grating at a position of maximum sensitivity. The lower response was elicited when the grating had been moved a quarter cycle (90°) away, to the null position. Contrast for both runs was 2.5%, and the time course of the temporal modulation of the contrast is indicated at the top of the column. Cell 12/1, on-centre, contralateral, LGN afferent recorded at cortex. The left hand column shows data from a Y geniculate cell in response to a 0.45 cycle per degree grating modulated on–off at 0.5 Hz. Contrast for both runs was 10%. Higher spatial frequencies were much less effective in exciting the cell, and response to on–off or alternating phase gratings were very similar when stimuli were equated for contrast. This cell had no null and at certain positions gave a burst of spikes when the grating went on and also when it was withdrawn. Cell 32/3, on-centre contralateral, recorded at LGN.

Fig. 2 Contrast sensitivity against position (spatial phase). *a*, An X geniculate cell; *b*, a Y geniculate cell. Sensitivity to elicit an average peak response of 20 impulses s^{-1} is plotted against position of the grating on the abcissa. Zero phase was set by the experimenter to give approximately maximum sensitivity. The ordinate, contrast sensitivity, is the reciprocal of the contrast required to give criterion response. The sign of the sensitivity was determined from the temporal phase of the response. Response at pattern on was considered positive; response at pattern off negative. A sine curve is fitted to the sensitivity function of the X cell. X cell 30/1 on-centre contra-lateral. Y cell 33/11 off-centre contralateral. Both recorded at LGN. The temporal frequency in both cases was 0.5 Hz; the spatial frequencies were 0.9 cycles per degree for the X cell and 0.45 cycles per degree for the Y cell.
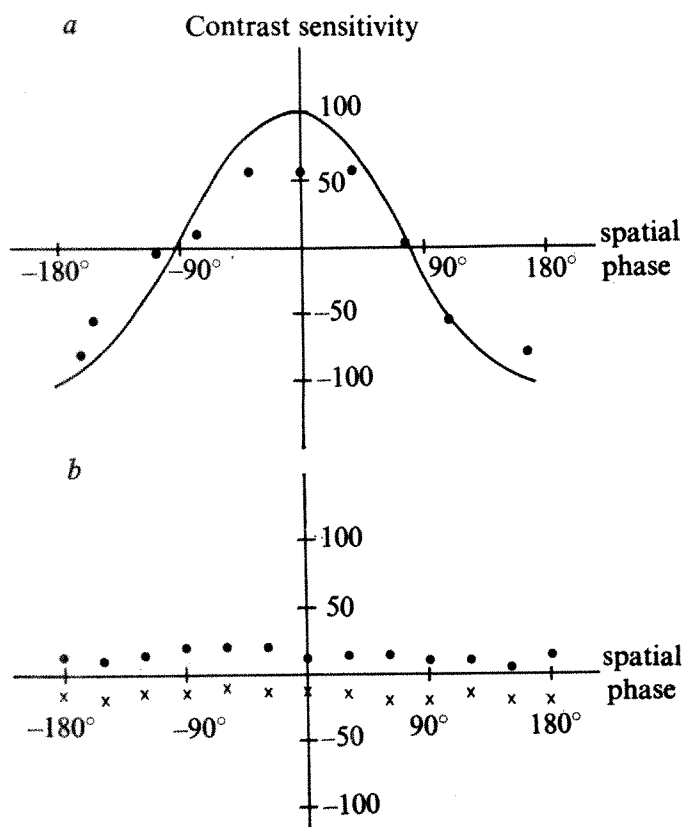
tional consequences of their parallel input to the visual cortex[5].

We used 18 X-like cells and 6 Y-like cells in 9 cats anaesthetised with urethane and paralysed with gallamine triethiodide. Action potentials were recorded from LGN cells and from LGN afferents to the striate cortex with tungsten-in-glass microelectrodes. LGN cells were distinguished from optic tract terminals by impulse wave form and polarity. Nerve impulses were averaged into post-stimulus time (PST) histograms with a computer. The cat looked at an oscilloscope screen through a contact lens with a 3-mm diameter artificial pupil. Refraction was determined with an ophthalmoscope and corrective lenses used when required.

Spatiotemporal patterns were created by means of a television-type raster display, with electronic modulation of the intensity on the oscilloscope screen. The patterns used for the experiments reported here were: (1) an on–off spatial sine grating introduced at a fixed contrast and then blanked, repetitively; (2) an alterating phase spatial sine grating, that is a grating which reversed contrast, or in other words shifted phase by a half cycle (180°), repetitively. For both kinds of patterns, mean luminance across the screen was always constant; only the spatial distribution of luminance varied with time. Pattern contrast was controlled by the experimenter. The mean luminance on the screen was 1 cd m^{-2}.

Some LGN cells had null positions when presented with on–off or alternating phase patterns. These cells are analogous to X retinal ganglion cells. An example is shown in Fig. 1. Shown on the right are the averaged responses of an X-type cell to an alternating phase grating when the grating was at the null position and when the grating was placed a quarter cycle (90° in spatial phase) away from the null. We also found Y-type cells in the cat LGN which had no null position for a grating regardless of spatial frequency and which at some positions produced bursts of impulses at pattern on and pattern off, or at both phases of pattern alternation, as shown for example in the left hand column of Fig. 1 (ref. 1).

We developed a quantitative measure of the strength of the null position of an LGN cell. This was the contrast sensitivity for a grating as a function of position. The reciprocal of the modulation depth required to elicit a criterion peak response, the contrast sensitivity, was plotted against position of the grating. (We called sensitivity for an on response positive and for an off response negative.) Such graphs for an X cell and a

Y cell are shown in Fig. 2. In this case, contrast sensitivity for the X cell at the null was more than a factor of thirty down from the peak sensitivity. For X cells, sensitivity was approximately a sinusoidal function of position of the grating. With linear spatial summation, a visual cell acts like a linear spatial filter for spatial patterns. Among other things, this means that the response to a sine grating must be a sinusoidal function of position, or spatial phase, of the grating in the visual field. The sensitivity profile of the cell need not be symmetrical to yield this result.

There was less variation of contrast sensitivity with position for Y cells, particularly at high spatial frequencies. Also, since at many positions a Y cell responds at both pattern on and pattern off, there are positive and negative contrast sensitivities at these points. Thus, two features of the graph of contrast sensitivity against position served to help classify a cell as either an X or Y cell: sinusoidal sensitivity curve with two nulls or a flatter sensitivity curve and the presence of single or double sensitivity values.

We found LGN cells which had null positions but very transient responses to standing contrast. Cell 30/1 in Fig. 2 was such a cell. Other X cells had a sustained component in their response, for example cell 12/1 in Fig. 1. Y cells with a sustained response also exist. Such results imply that the X/Y classification[1] and the "sustained/transient" classification of Cleland et al.[2,3] (see also Hoffman et al.[4]) may not be identical, at least for LGN cells. The classification of visual cells into two groups, X and Y, based on their spatial interactions is as clear and unambiguous, in the LGN as in the retina. Our work shows that the X/Y retinal dichotomy, characterised by the degree of linearity of spatial summation, is preserved all the way to the visual cortex.

ROBERT SHAPLEY
SAUL HOCHSTEIN

Rockefeller University,
New York, New York 10021

1 Enroth-Cugell, C., and Robson, J. G., *J. Physiol., Lond.*, **187**, 552 (1966).
2 Cleland, B. G., Dubin, M. W., and Levick, W. R., *J. Physiol., Lond.*, **217**, 473–496 (1971).
3 Cleland, B. G., Levick, W. R., and Sanderson, K. J., *J. Physiol., Lond.*, **228**, 649–680 (1973).
4 Hoffman, K. P., Stone, J., and Sherman, S. M., *J. Neurophysiol.*, **35**, 518–531 (1972).
5 Stone, J., and Hoffman, K. P., *Brain Res.*, **32**, 460–466 (1971).

Sporulation-inducing factor in slime mould *Physarum polycephalum*

THE highly synchronous sporulation of *Physarum polycephalum* is an attractive model of cell differentiation. Virtually all of the nuclei in a starved plasmodium proceed in step through the sporulation process in response to a well defined stimulus, light[1-4]. The regulatory mechanisms of this differentiation are unknown but several studies have been presented on the conditions necessary for sporulation. These have been reviewed by Sauer[5], who concludes that two elements are necessary for sporulation, substrate and light. These must be applied sequentially. The substrate requirement is for starvation in the dark for at least 4 d in the presence of niacin and niacinamide[4]. The light requirement is for at least 4 h illumination by a 40 W fluorescent light. Sauer also cites unpublished results which suggest that the effect of illumination is a local one which cannot be transferred to an unilluminated, starved plasmo-

Table 1 Induction of sporulation by microinjection

Injected substance	Sporulation frequency No. of plasmodia sporulating / No. tested	(%)
None	0/4	0
Distilled water	0/10	0
Unilluminated plasmodial contents	0/14	0
Illuminated plasmodial contents	11/18	61
UM10 filtrate	3/5	60
UM10-retained	7/10	70
NaCl (0.06 M)	33/113	29
KCl (0.06 M)	4/33	12
Na_2SO_4 (0.03 M)	2/19	11
$(NH_4)_2SO_4$ (0.03 M)	2/16	13

Results are the means of several experiments. Macroplasmodia were established in 9-cm Petri dishes containing sporulation medium (1) in 2% agar. After starvation on this medium for 6 d in the dark at 21 °C, these macroplasmodia were microinjected with 10 μl of the indicated substances and immediately placed back in the dark at 21 °C. Illuminated macroplasmodia were prepared in the same manner except that, at the end of the starvation period, they were placed 6 cm from a 40 W fluorescent light bulb for 6 h. A fan kept the temperature of the dishes below 24 °C. Clarified extracts were prepared by homogenising ten macroplasmodia in 1.5 ml deionised water and centrifuging for 10 min at 26,000g in a Beckman J21 centrifuge. Extracts were then ultrafiltered with Amicon UM10 membranes.

dium or even from the illuminated half to the unilluminated half of the same plasmodium.

In this report we present results which show that the sporulation-inducing effects of illumination can indeed be transferred to an unilluminated, starved plasmodium by microinjection. Furthermore, the requirement for light can be circumvented altogether by the injection of a small amount of salt solution into a starved plasmodium.

The top part of Table 1 shows that a small quantity of plasmodial contents from an illuminated, starved plasmodium will induce sporulation in a starved, unilluminated plasmodium. The sporulation thus induced is usually complete 20 h after injection, but it sometimes takes up to 48 h. Thus, this process is slower than sporulation induced by light, which takes 13-15 h, but it is homogeneous, involving the entire plasmodium synchronously and is morphologically indistinguishable from that observed in an illuminated slime mould. This effect is not produced by injecting an equivalent amount of water, pricking with a micropipette, placing the plasmodium on the dissecting microscope stage in the light for 5 min (the duration of the injection process) or by injecting an equivalent amount of plasmodial contents from an unilluminated, starved plasmodium.

The approximate size of the inducing factor has been determined by ultrafiltration with Amicon membranes. The middle part of Table 1 shows that a factor passes through a UM10 filter and therefore has a molecular weight of less than 10,000. The active substance in the UM10-retained fraction could be the same as that which passes through the filter, as filtration was not taken to completion. It is also possible that there is another, larger component present in the retained fraction.

The bottom part of Table 1 shows that a variety of salts, injected into a starved, unilluminated plasmodium also induced sporulation. The concentration of the cation was held constant in each case. The effect of NaCl seems most pronounced, although the differences may not be significant.

We are unable at this time to determine whether illumination and salt operate through the same or distinct mechanisms of sporulation control. It is evident, however, that there is some differentiation-inducing factor in illuminated slime mould which can be transferred to an unilluminated slime mould. Elucidation of the nature of the triggering

substance(s) may thus help to explain the mechanism of control of differentiation in this organism.

We thank Ms S. Vuturo for assistance. This work was supported by the Research Corporation, the National Institute of General Medical Sciences and the University of Kansas General Research Fund.

WILLIAM M. WORMINGTON
CHONG G. CHO
ROBERT F. WEAVER

Department of Biochemistry,
McCollum Laboratories,
University of Kansas,
Lawrence, Kansas 66044

1 Daniel, J. W., *Cell Synchrony* (edit. by Cameron, I. L., and Padilla, G. M.), 117–152 (Academic, New York, 1966).
2 Gray, W. D., *Am. J. Bot.*, 25, 511–522 (1938).
3 Daniel, J. W., and Rusch, H. P., *Fedn Proc.*, 17, 434 (1958).
4 Daniel, J. W., and Baldwin, H. H., *Meth. Cell Physiol.*, 1, 9–41 (1964).
5 Sauer, H. W., *Microb. Different.*, 23, 380–700 (1973).

Inhibition of fungal growth by wheat germ agglutinin

GROWTH of fungal hyphae is the result of a complex and poorly understood process of cell wall synthesis and extension, that is restricted to the hyphal apex[1-3]. In fungi with chitin–glucan hyphal walls, such as the Deuteromycetes *Trichoderma viride* and *Fusarium solani*[1], hyphal extension and septa formation involve the synthesis of chitin in hyphal tips and septa[4]. We therefore studied the effect on such fungi of wheat germ agglutinin (WGA), a lectin which interacts specifically with chitin oligomers[5,6].

Although interactions of plant lectins with a variety of animal cells and with different microorganisms are well documented (for reviews see refs 7 and 8), no information on their effect on fungi is available. We have found that WGA inhibits growth and spore germination of *T. viride*. These findings suggest a role for the lectin in nature, and provide further insight into the mechanism of fungal cell wall growth.

The binding of WGA to *T. viride* Pers. ex Fries isolate No. M 2042, was studied using fluorescein isothiocyanate-conjugated WGA (FITC-WGA). The lectin binds exclu-

sively to hyphal tips and septa, whereas most of the hyphae are not labelled (Fig. 1). The chitin in the septa and tips seems to be accessible to WGA whereas in the mature regions of the hyphae, the overlayering of chitin by glucans may prevent WGA binding. Effective labelling could be observed even after only a 10 s exposure of *T. viride* to a solution of 15 μg per 0.1 ml FITC-WGA. No labelling was observed when the colonies were preincubated with non-fluorescent WGA or when treated with FITC-WGA preincubated (30 min at 37 °C) with chitotriose (2 mM), a specific inhibitor of the lectin[5].

[3]H-acetate has been used as a morphogenetic marker of intact *T. viride* colonies[4]. Short pulse labelling of colonies in the course of differentiation clearly labels the growing zones of the hyphae. Although acetate is not incorporated specifically into chitin, it is noteworthy that in young hyphae of *T. viride* chitinase removed all label from the cell wall after short ^{3}H-acetate incorporation, thus demonstrating that most of the cell wall synthesis during short pulses is confined to production of chitin. As we had observed that WGA binds to hyphal tips, we studied the effect of the lectin on hyphal growth as indicated by ^{3}H-acetate incorporation. Figure 2 demonstrates that as little as 125 μg WGA per 0.1 ml growth solution markedly inhibits ^{3}H-acetate incorporation into hyphal tips, which is an indication of arrest of growth[4]. The incorporation of ^{3}H-acetate was not affected in experiments in which WGA, preincubated with chitotriose (2 mM), was added to the colonies.

Fig. 1 Microscopic appearance of *T. viride* hyphae treated with WGA labelled with fluorescein isothiocyanate. WGA was purified by affinity chromatography[9] and conjugated with fluorescein isothiocyanate[10]. *T. viride* was cultured on 25 × 75 mm glass slides with frosted ends[4]. After 30–40 h growth in dark, FITC-WGA (50 μg in 0.1 ml phosphate buffered saline, pH 7.4) was spread over half of the colony and allowed to interact for 10 min at room temperature. Slides were then washed with saline and observed with a standard RA Zeiss fluorescence microscope. Exciter filter BG-12 was used in the path of the excitation light and barrier filter No. 53 in the path of the emitted light. *a*, Binding to hyphal tips; *b*, binding to septa. Bars represent 10 μm.
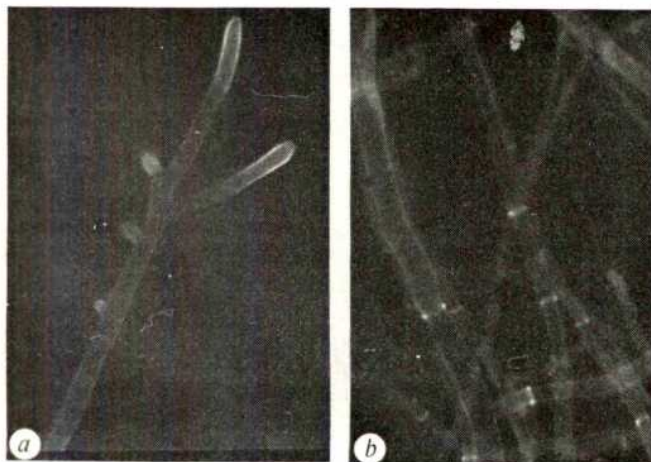
Fig. 2 Microautoradiographs of *T. viride* hyphae. Colonies of *T. viride* grown on slides[4] were overlayered with WGA (30–500 μg in 0.1 ml minimal medium consisting of 10 g glucose, 0.5 DL-alanine, 1 g KH$_2$PO$_4$ and 0.5 g MgSO$_4$ in 1 l water). After incubation of 10 min at 25 °C, ^{3}H-sodium acetate (18 Ci mmol^{-1}; 4 μCi in 0.1 ml minimal medium) was added on top of the WGA solution, and incorporation of label was allowed to proceed for 10 min. The slides were washed, fixed and processed for autoradiography as described[4]. *a*, Hyphae from a colonial front after incubation for 10 min with ^{3}H-sodium acetate, normal optics. Note heavy labelling of hyphal tips and septum. *b*, Hyphae from a colony which was preincubated with 250 μg WGA and then labelled and photographed as in *a*. Note decrease in labelling at hyphal tip. Bars represent 20 μm.

To obtain a more quantitative evaluation of the inhibition of ^{3}H-acetate incorporation by WGA, we studied the effect with *T. viride* cultured by shaking in suspension. WGA markedly inhibited ^{3}H-acetate incorporation into a suspension of young hyphae (60% inhibition at 0.8 mg ml^{-1} WGA), whereas the incorporation of ^{3}H-leucine was practically unaffected (Fig. 3*a*). In these experiments chitotriose (2 mM) also inhibited the effect of WGA.

To observe this possible inhibition of hyphal growth macroscopically, Petri dishes (9 cm diameter), containing potato dextrose agar were inoculated with *T. viride* and
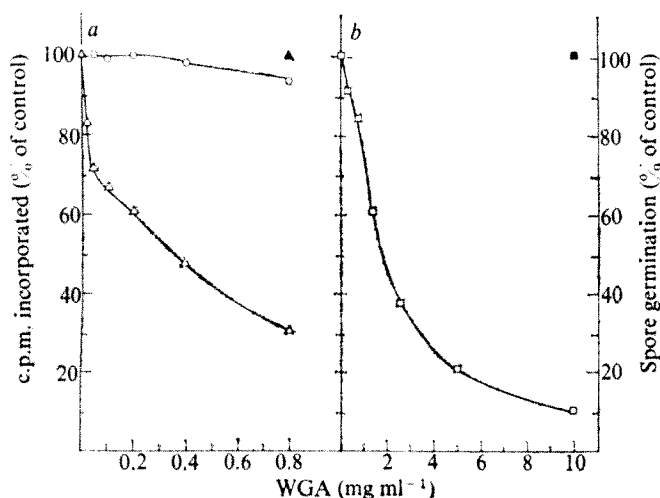
Fig. 3 *a*, Inhibition by WGA of ³H-sodium acetate incorporation into young hyphae. A suspension of young hyphae was obtained by allowing *T. viride* spores to germinate by shaking for 18 h in minimal medium at 25 °C. Aliquots (2 ml) of this suspension (0.27 mg dry weight ml⁻¹) were added to WGA solution (50–2,000 µg in 0.5 ml minimal medium) in 10 ml Ehrlenmeyer flasks. After 15 min of shaking at 25 °C, ⁴H-sodium acetate (Δ18, Ci mmol⁻¹, 4 µCi in 0.1 ml minimal medium) was added to each flask and the flasks were shaken for a further 5 min. The pulse labelling was terminated by addition of 3 ml cold solution CCl₃COOH (10% w/v) and the flasks kept in the cold for 15 min. Suspension was filtered over GF/C glass filters (Whatman) and washed with 20 ml 1 N acetic acid. Filters were dried for 2 h at 100 °C and the radioactivity remaining counted in toluene scintillation liquid with an efficiency of 52%, using a Packard Tri-Carb scintillation spectrometer. In parallel experiments, the incorporation of ³H-leucine (19 Ci mmol⁻¹, 4 µCi in 0.1 ml) was followed in the same conditions. Percentage inhibition was calculated from the ratio of the radioactivity incorporated in the presence of WGA to that incorporated in its absence. ▲, Obtained when WGA was preincubated (30 min at 37 °C) with chitotriose (2 mM). *b*, Inhibition by WGA of *T. viride* spore germination. WGA (10–300 µg in 15 µl minimal medium) was mixed with *T. viride* spores (15 µl suspension of 0.27 mg dry weight per ml in minimal medium) and the spores were allowed to germinate for 18 h at 25 °C. Aliquots (25 µl) were spread on a microscope slide and germination was observed under the microcope. Percentage germination was calculated after counting 1,000 spores germinated in the presence and absence of WGA. ■, Germination in presence of WGA and 2 mM chitotriose.

kept in the dark at 25 °C. When the colonies reached a diameter of 4 cm (after 2 d), they were exposed to laboratory light; three wells were drilled in the agar, 1 cm from the front of the colony (3 cm from the centre of the plate) and filled with WGA (30–500 µg in 50 µl minimal medium) and the colonies were allowed to grow (4 d) until they covered the dish. Exposure to light induced conidiation[11] and green rings of conidia marked the pattern of the colonies' extension. As little as 60 µg WGA in a well inhibited hyphal extension (Fig. 4*a* and *b*). The area of the clear zone around the wells, where growth was inhibited, was concentration-dependent. The specificity of the effect is indicated by the normal growth of the fungus when WGA was placed in wells in the presence of the WGA-inhibitors, chitotriose (2 mM) or teichoic acid of *Staphylococcus aureus* H (0.6 mM), (Fig. 4*c*). The latter is a very potent inhibitor of WGA (ref. 12).

It has been shown that the cell wall of hyphal tubes emerging out of germinating spores of the fungal group V (ref. 1), to which *T. viride* belongs, contain chitin[3]. Indeed, a marked inhibition of spore germination was observed when WGA was mixed with spores of *T. viride* (Fig. 3*b*). The effect of WGA in inhibiting the germination of the spores of these fungi seems to be the result of its binding to the chitin at the tip of the emerging hyphal tube rather than to the spore coat, as no binding of FITC-WGA to the spores could be observed. Furthermore, the spores were not agglutinated by WGA.

The evidence presented above indicates that binding of WGA to hyphal tips inhibits chitin synthesis, hyphal growth and spore germination. Although the mechanism of apical growth is poorly understood, it has been suggested that the addition of newly synthesised chitin for extension of the hyphal cell wall and the onset of germination in spores, require the cleavage of pre-existing chitin in the hyphal tip by selective lysis[3]. Binding of the multivalent WGA (refs 13 and 14), to the chitin at the hyphal tip may result in cross-linking of the latter and thus inhibit the extension process. The perturbation of the delicate balance between wall synthesis and wall lysis which presumably controls apical wall growth[3] may cause the arrest of chitin synthesis. The effect of WGA seems to be of a fungistatic nature as it does not cause inhibition of leucine incorporation during short pulses (Fig. 3*a*).
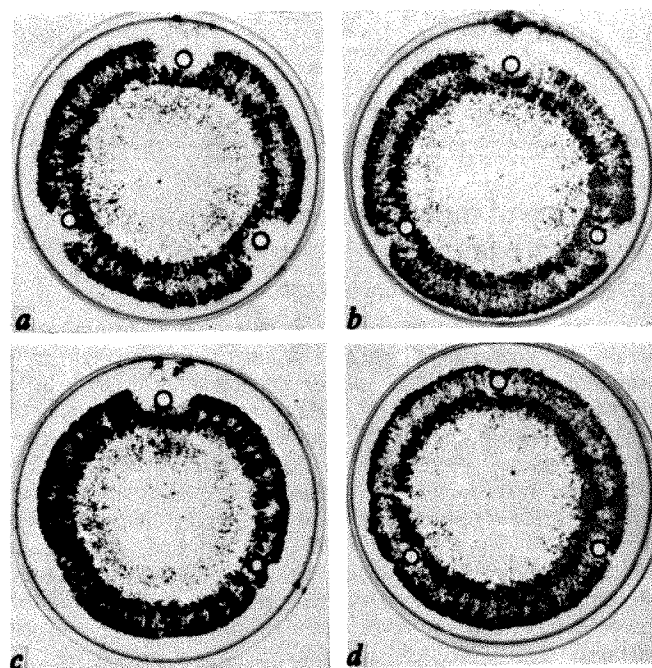
Fig. 4 Inhibition of conidiation and arrest of *T. viride* hyphal extension by wheat germ agglutinin. *T. viride* was grown on potato dextrose agar in Petri dishes. When the colonies reached a diameter of 4 cm, WGA was placed in wells drilled in the agar 1 cm from the front of the colony and the colonies were allowed to grow further. The wells on top, right, and left contained: *a*, 500 µg, 250 µg and 125 µg WGA, in 50 µl minimal medium, respectively; *b*, 125 µg, 60 µg and 30 µg WGA respectively; *c*, top well contained 250 µg WGA, right-hand well contained the same amount of lectin together with 1 mg purified teichoic acid of *S. aureus* H, left-hand well contained 250 µg WGA and 2 mg chitotriose; *d*, all the wells contained minimal medium (50 µl).

A possible role of plant lectins in attaching nitrogen-fixing bacteria to plant roots has been proposed[15,16]. In view of the experiments described in this paper, as well as our finding that FITC-WGA binds to the hyphal tips of *Fusarium solani* (Mart.) Sacc. and inhibits ³H-acetate incorporation into their cell wall, it is tempting to suggest a role for this lectin in nature. It is possible that WGA protects wheat against chitin-containing phytopathogens during seed imbibition, germination and early seedling growth. The role of lectins in protecting plant seeds in which their concentration is usually high, may be a general one. Accordingly, lectins with sugar specificities which differ from that of WGA may inhibit the growth of other soil microorganisms, the surface of which are covered by polysaccharides such as glucans, galactans, mannans, or various heteropolysaccharides[1].

We thank Mr C. Tabbak for assistance. This work was

supported in part by the US–Israel Binational Science Foundation.

DAVID MIRELMAN
ESRA GALUN
NATHAN SHARON
REUBEN LOTAN

Departments of Biophysics and Plant Genetics,
The Weizmann Institute of Science,
Rehovot, Israel

1 Bartnicki-Garcia, S., *A. Rev. Microbiol.*, **22**, 87-108 (1968).
2 Girbardt, M .,*Protoplasma* (Wien), **67**, 413-441 (1969).
3 Bartnicki-Garcia, S., *Symp. Soc. gen. Microbiol.*, **23**, 245-267 (1973).
4 Galun, E., *Archs Mikrobiol.*, **86**, 305-314 (1972).
5 Allen, A. K., Neuberger, A., and Sharon, N., *Biochem. J.*, **131**, 155-162 (1973).
6 Lotan, R., and Sharon, N., *Biochem. biophys. Res. Commun.*, **55**, 1340-1346 (1973).
7 Sharon, N., and Lis, H., *Science*, **177**, 949-959 (1972).
8 Lis, H., and Sharon, N., *A. Rev. Biochem.*, **42**, 541-574 (1973).
9 Lotan, R., Gussin, A. E. S., Lis, H., and Sharon, N., *Biochem. biophys. Res. Commun.*, **52**, 656-662 (1973).
10 Clark, H. F., and Shepard, C. C., *Virology*, **20**, 642-644 (1965).
11 Galun, E., and Gressel, J., *Science*, **151**, 696-698 (1966).
12 Lotan, R., Sharon, N., and Mirelman, D., *Eur. J. Biochem.* **55**, 257-262 (1975).
13 Nagata, Y., and Burger, M.M ., *J. biol. Chem.*, **249**, 3116-3122 (1974).
14 Privat, J. P., Delmotte, F., and Monsigny, M., *FEBS Lett.*, **46**, 224-228 (1974).
15 Hamblin, J., and Kent, S. P., *Nature new Biol.*, **245**, 28-30 (1973).
16 Bohlool, B. B., and Schmidt, E. L., *Science*, **185**, 269-271 (1974).

Inhibition of mobility of cell-surface receptors by factors which mediate specific cell–cell interactions

PREVIOUS studies on embryonic chick neural retina cells[1] have demonstrated that cells in intact tissues or single cells prepared by mechanical dispersion have relatively little ability to redistribute their surface receptors into caps. In contrast, cells prepared by trypsinisation and dissociation of intact tissues were found to exhibit lateral redistribution of surface receptors even in the absence of a multivalent ligand. After 4 h culture, however, cells dissociated by trypsinisation have lost the capability for both spontaneus and lectin-induced rearrangements of surface components. Mobility of cell-surface receptors thus seems to be related to, or determined by, trypsin-labile components at the cell surface. One kind of component which seems to be trypsin labile is also important in intercellular recognition and adhesion[2,3]. We now report that the binding of tissue-type specific cell-surface ligands which seem to mediate recognition and adhesion causes inhibition of directed rearrangement of plant lectin receptors in the plane of the membrane.

Procedures for collection of aggregation-promoting supernatant solutions (APMs), retina aggregation promoting material (RAPM) and cerebral lobe aggregation promoting material (CLAPM) were as described previously[4]. The assay for redistribution of fluorescent-labelled lectin receptors on the surface of 10-d embryonic chick neural retina cells freshly dissociated from tissues by trypsinisation was carried out as described previously[1]. The same procedure was used for 10-d cerebral lobe cells. Fluorescein-isothiocyanate conjugated concanavalin A (FITC–con A), wheat germ agglutinin (FITC–WGA) and soybean agglutinin (FITC–SBA) were purchased from Miles. Cells labelled with fluorescent lectin were examined under a Zeiss Universal microscope using an epifluorescence system and ×40 oil-immersion objective. Protein determinations on dialysed aliquots of RAPM and CLAPM were carried out by the method of Lowry *et al.*[5].

As shown in Fig. 1, RAPM inhibits cap formation of FITC–con A receptors on trypsin–dissociated 10-d chick neural retina cells. The effect depends on concentration with a plateau value at approximately 80% inhibition at RAPM concentrations greater than 25 μg ml protein. Added CLAPM or foetal calf serum (Gibco) have a
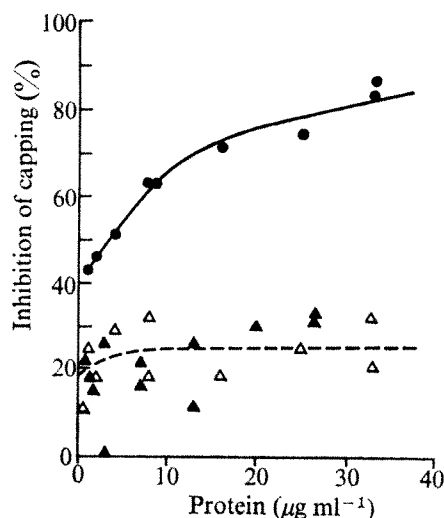
Fig. 1 Effect of APMs and foetal calf serum on cap formation of con A receptors in 10-d chick neural retina cells. Cells (20 × 10^6) prepared by trypsinisation were incubated with 60 μg FITC–con A in 1 ml Eagle's basal medium for 20 min at 0°C, followed by incubation for 60 min at 22°C with or without additives, pelleted at 200g for 5 min, and fixed in 2% glutaraldehyde (pH 7.4) for 30 min at 0°C. ●, RAPM; ○, CLAPM; ▲, foetal calf serum.

maximal effect of approximately 25% inhibition of capping over the same protein concentration range. None of these preparations had any effect on patch formation.

The inhibitory effect of RAPM on surface receptor mobility is not limited to the con A receptors. Figure 2 illustrates that receptors for both FITC–WGA and FITC–SBA are also rendered increasingly immobile after treatment with various concentrations of RAPM, although the maximal inhibition of capping by RAPM only reaches a level of approximately 50% for these receptors. As is the case for the con A receptors, CLAPM causes much less inhibition of capping of WGA and SBA receptors, with maximal inhibition of 15–20%.

To examine the tissue-type specificity of the effect of APMs on lectin receptor mobility, 10-d chick cerebral lobe cells were used in otherwise identical experiments. In this case, CLAPM inhibits cap formation of con A receptors in a concentration-dependent manner to a level of approximately 80%. The maximal inhibition obtained with

Fig. 2 Inhibition by APMs of cap formation of wheat germ agglutinin (●, ○, solid lines) and soybean agglutinin (▲, △, dashed lines) receptors in 10-d chick neural retina cells. Experimental conditions as described in Fig. 1. ●, ▲, RAPM; ○, △, CLAPM.
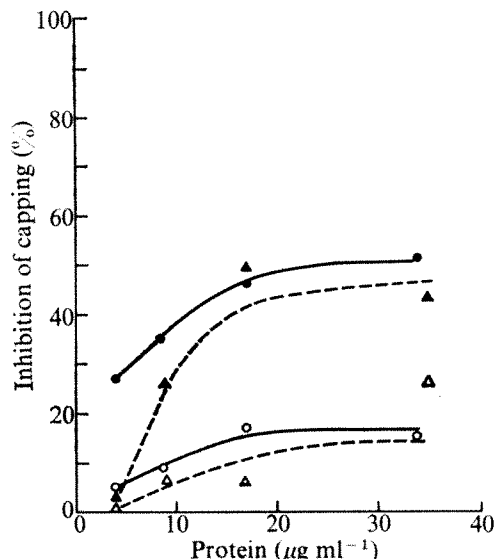
RAPM on cerebral lobe cells is 25% (Fig. 3). These results suggest that RAPM and CLAPM are able to specifically immobilise receptors on the cell type from which they originate.

Control of the mobility of one surface component by another has also been reported in lymphocytes[6,7]. In this system, incubation of cells with concentrations of con A greater than 5 μg ml^{-1} causes inhibition of cap formation of surface immunoglobulin molecules. Drugs which are thought to disrupt microtubules, however, were able to reverse the inhibition by con A. To examine whether inhibition of the mobility of lectin receptors by RAPM is an analogous situation, cytochalasin B (Aldrich, 20 μg ml^{-1} in dimethyl sulphoxide) or colchicine (40 μg ml^{-1}, Sigma) were added to retina cells being assayed for receptor redistribution with FITC–con A. No reversal of the inhibition by RAPM was observed. Moreover, we have previously observed[1] that treatment with these drugs does not overcome to the inhibition of con A receptor mobility in the presence of excess con A.
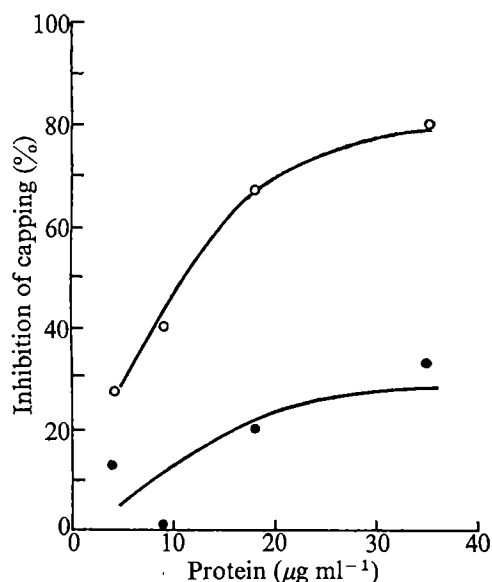
Fig. 3 Inhibition by APMs of cap formation of con A receptors in 10-d chick cerebral lobe cells. Experimental conditions as described in Fig. 1. ●, RAPM; ○, CLAPM.

It has previously been demonstrated in this laboratory that binding of RAPM to retina cells depends on a terminal N-acetyl-galactosamine residue, whereas binding of CLAPM to cerebral lobe cells depends on a terminal mannosamine residue[8]. To establish a positive correlation between binding activity and inhibition of surface component redistribution activity, RAPM was treated with purified N-acetyl-hexosaminidase (Miles) and CLAPM with purified α-mannosidase (Miles). Enzyme-treated RAPM preparations have a decreased ability to inhibit con A receptor mobility on neural retina cells that almost exactly corresponds to the loss of binding activity (Table 1). Similarly, CLAPM treated with α-mannosidase shows a parallel decrease in ability both to bind to cells and to inhibit con A receptor mobility of cerebral lobe cells (Table 1). Thus, by the criteria of both specificity of action and behaviour after enzyme treatment, it seems that the portion of the APM preparation which has binding activity is very similar to, or identical with, the portion which inhibits surface receptor mobility.

Taken together, these results suggest that supernatant preparations from cultures of 10-d chick neural retinae or cerebral lobes enhance reaggregation of dissociated single cells in a tissue-type specific fashion[3,9], specifically bind to such cells[4], and are also able to specifically induce an alteration in some membrane-related parameter, the effect of

Table 1 Comparison of loss of binding activity with loss of inhibition of lectin receptor redistribution activity after glycosidase treatment

APM	Preparation	Loss of binding activity (%)	Loss of inhibition of lectin receptor redistribution activity (%)
RAPM	1	20	14
	2	40	35
	3	60	70
CLAPM	1	10	26
	2	50	45
	3	70	63

RAPM was incubated in 0.05 M sodium acetate buffer (pH 4.0) at 37 °C for 30 min with 0.10, 0.25, and 0.45 U ml^{-1} purified N-acetyl-hexosaminidase (Miles) for preparations 1, 2, and 3, respectively. Enzyme-treated RAPM was boiled for 10 min and dialysed overnight against Tyrode's solution. CLAPM was treated with purified α mannosidase at concentrations of 0.01, 0.03, and 0.04 U ml^{-1} for preparations 1, 2, and 3, respectively, using the conditions above with the exception that the incubation was for 60 min. Boiled enzyme controls were done in each experiment. Binding to cells was carried out as described previously[4]. Experimental conditions for the assay of inhibition of lectin receptor mobility were as described in the legend to Fig. 1. RAPM was assayed on 10-d chick neural retina cells, CLAPM on 10-d chick cerebral lobe cells.

which is to inhibit directed rearrangements of surface receptors into caps. As receptors for three different lectins which have been shown to redistribute independently in the plane of the membrane[1] are all affected in basically the same manner by APMs, we infer that the inhibition is a general one, affecting most or all of the cell-surface components.

Control of cell-surface topography by ligands specific for tissue type may be of fundamental importance to the processes of cell recognition, adhesion and histotypic rearrangement. Whether or not neighbouring cells form stable contacts may depend, at least partially, on the distribution of surface components important for recognition and adhesion. The action of such ligands at the surface of each cell may be to maintain the surface of appropriate neighbouring cells in a configuration most suitable for stable contacts. A similar hypothesis has been advanced by Bennett et al.[10] which suggests that epigenetic control of cell-surface architecture is an important element in determination and morphogenesis. The combined use of general surface markers, such as lectin probes, with tissue-type-specific surface markers, such as APMs, should enable exploration of this hypothesis in normal morphogenetic contexts.

JIM MCDONOUGH
JACK LILIEN

Department of Zoology,
University of Wisconsin,
Madison, Wisconsin 53706

1 McDonough, J., and Lilien, J , *J. Cell Sci.* (in the press).
2 Roth, S., *Devl Biol.*, 18, 602 (1968).
3 Lilien, J., in *Curr. Top. devl Biol.*, 4, (edit. by Moscona, A. A., and Monroy, A), 169 (Academic, New York, 1969).
4 Balsamo, J., and Lilien, J., *Proc. natn. Acad. Sci. U.S.A.*, 71, 727 (1974).
5 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. biol. Chem.*, 193, 265 (1951).
6 Edelman, G. M., Yahara, I., and Wang, J. L., *Proc. natn. Acad. Sci. U.S.A.*, 70, 1442 (1973).
7 Unanue, E. R., and Karnovsky, M. J., *J. exp. Med.*, 140, 1207 (1974).
8 Balsamo, J., and Lilien, J., *Biochemistry*, 14, 167 (1975).
9 Garber, B. B., *In Vitro*, 8, 167 (1972).
10 Bennet, D , Boyse, E. A., and Old, L. J., in *Cell Interactions, Proceedings of the Third Lepetit Colloquium* (edit. by Silvestri, L. G.), 247 (American Elsevier, New York, 1972).

Stimulation by cyclic GMP of lymphocytes mediated by soluble factor released from adherent cells

THE activation of lymphocytes by mitogens is a complex phenomenon which can be subdivided into reactions occurring on the surface of the cells[1] followed by a chain of intracellular reactions caused by biochemical alterations in the membrane[2]. With respect to the mediation of the mitogenic signal from the membrane to the nucleus it has been claimed that 3',5'- cyclic GMP either directly fulfils a role as a 'second messenger'[3], or that a 3',5'-cyclic GMP-dependent step is involved in the chain of intracellular reactions culminating in cell mitosis[3,4]. These assumptions are based on the facts that after the addition of lectins to peripheral white blood cells depleted of granulocytes and monocytes, an increased intracellular concentration of 3', 5'-cyclic GMP was detected[3] and that 3',5'-cyclic GMP added exogenously activated DNA synthesis in spleen and thymus cells *in vitro*[4-6]. The prerequisites for the validity of the above mentioned hypothesis, however, are that the 3',5'-cyclic GMP levels are indeed increased in all cells entering the mitotic phase and that 3',5'-cyclic GMP added exogenously acts directly on the lymphocytes, without the participation of other cells.

Here we present results which show that stimulation of DNA synthesis in lymphocytes by exogenously added 3',5'-cyclic GMP requires the presence of adherent cells, and that peritoneal cells of mice, rich in macrophages, release a non-dialysable factor which stimulates DNA synthesis in lymphocytes if cultured in presence of 3',5'-cyclic GMP or with various mitogens.

The results of a typical experiment are summarised in Table 1. Spleen cells of BALB/c mice depleted of glass-adherent cells gave a smaller response to 3',5'-cyclic GMP than a normal spleen cell population as measured by thymidine incorporation. Addition of peritoneal cells to the non-adherent cell population restored or even enhanced the response of the cells. Removal of adherent cells, however, does not diminish markedly the response of the cells to either 2',3'-cyclic GMP, lipopolysaccharide (LPS), or phytohaemagglutinin (PHA). The response of thymocytes to 3',5'-cyclic GMP was enhanced in the presence of peritoneal cells. These results indicate that the stimulatory activity of 3',5'-cyclic GMP on DNA synthesis in spleen cells and in thymocytes is at least partially mediated by a process dependent on an adherent cell population.

To test the possibility that lymphocyte activating factor(s) released from peritoneal cells[7] may be involved in these effects,

we cultured peritoneal cells of BALB/c mice with various cyclic nucleotides. Thymocytes or spleen cells were then cultured in a medium containing various amounts of supernatants derived from peritoneal cells incubated with the various cyclic nucleotides. The results of a typical experiment are shown in Table 2. As measured by thymidine incorporation only supernatants derived from 3',5'-cyclic GMP-treated peritoneal cells stimulated DNA synthesis in thymocytes or spleen cell cultures. Peritoneal cell supernatants from non-treated cultures failed to stimulate spleen cells and only marginally stimulated (stimulation index less than 1.8) thymocyte cultures. The response to the active supernatants was dose dependent, and optimum stimulation was obtained when the cells were incubated in the presence of supernatants at a final concentration of 10% (data not shown). 2',3'-cyclic GMP at a concentration of 10^{-3} M, which has been reported to induce DNA synthesis in lymphoid cells including thymocytes[8], or 3',5'-cyclic AMP, a compound which antagonises the mitogenic activity of 3',5'-cyclic GMP at the concentration used[4], both failed to produce an active supernatant.

Dialysed supernatants of 3',5'-cyclic GMP-treated cultures as tested on thymocyte cultures had markedly more activity (Table 2), indicating the presence of a low molecular weight inhibitory principle, probably identical with that found in serum-stimulated peritoneal exudate cell culture fluids[9]. Similar stimulatory activities could be obtained by incubation of peritoneal cells with LPS or PHA (data not shown) indicating that the stimulatory principle released by 3',5'-cyclic GMP may be similar to, or identical with, that described[7,9] as lymphocyte activation factor.

Most of the biological activities of 3',5'-cyclic GMP have been reported to require Ca^{2+} (ref. 10). The release of the stimulatory principle from peritoneal cells by 3',5'-cyclic GMP, however, seems to be independent of Ca^{2+} (Table 2). Ethyleneglycol-*bis*-(2-amino-ethylether)-N,N'-tetraacetic acid (EGTA), a chelator for Ca^{2+} added at a concentration (0.6 mM) which completely inhibits the response of lymphocytes to mitogens[11,12], including the effect of 3',5'-cyclic GMP[4], failed to abolish the release of the active principle from peritoneal cells by 3',5'-cyclic GMP. Addition of 3',5'-cyclic AMP at concentrations which inhibit completely the stimulatory activity of 3',5'-cyclic AMP, PHA or LPS[4] on spleen cells also failed to abolish the release of lymphocyte activating factor by 3',5'-cyclic GMP from peritoneal cells.

In conclusion, the action of 3',5'-cyclic GMP on lymphocytes is not exclusively a direct one and the stimulation of lymphocytes by 3',5'-cyclic GMP is at least in part mediated by

Table 1 The stimulatory activity of peritoneal cells on response of non-adherent spleen cells and of thymocytes to 3',5'-cyclic GMP

Responder cells	None	3',5'-cyclic GMP (5 × 10⁻³ M)	2',3'-cyclic GMP (10⁻³ M)	PHA (10 μg ml⁻¹)	LPS (50 μg ml⁻¹)
			c.p.m. per culture		
Spleen	119	1,191	3,187	4,262	974
Non-adherent spleen	79	238	2,379	5,487	818
Non-adherent spleen + 2% PC	355	721	2,631	13,952	1,285
Non-adherent spleen + 5% PC	357	1,234	2,978	16,038	1,250
Non-adherent spleen + 10% PC	377	1,839	3,137	16,066	1,197
Thymocytes	48	157	388	224	84
Thymocytes + 10% PC	52	546	447	· 493	178

Spleen or thymus cell suspensions of BALB/c mice in a total volume of 0.15 ml (2 × 10⁶ cells ml⁻¹) were cultured in Microtitre plates M24AR, system Cooke (C. A. Greiner, Nürtingen, Germany) in an atmosphere of 5% CO_2 in air at 37 °C for 48 h in RPMI1640 medium supplemented with 5% fresh heat-inactivated human serum, 2 mM L-glutamine, 100 U penicillin and 100 μg streptomycin ml⁻¹ (culture medium). ³H-thymidine (0.1 μCi; 2 Ci mM⁻¹; Radiochemical Centre, Amersham) was added to the cultures for the last 4 h of incubation period. Cells were collected on glass fibre filters using a Skatron multiple cell culture collecter. Incorporation of ³H-thymidine into the nuclear DNA was then determined as described previously[4]. Non-adherent spleen cells, containing less than 1% macrophages, were obtained using a glass bead column as described previously[13]. Peritoneal cells (PC) of BALB/c mice were obtained by injecting 5 ml culture medium intraperitoneally and aspirating the fluid from the peritoneal cavity 5 min later. The cell population obtained consisted of ~75% monocytes, 10% polymorphs, and 15% lymphocytes. Cyclic nucleotides were obtained from Boehringer, Mannheim; LPS (lipopolysaccharide of *E. coli* 055:B5) and PHA from Difco, Detroit. Results represent arithmetic means of c.p.m. detected in triplicate cultures, s.e. less than 10%. As determined by separate experiments the highest number of PC (3 × 10⁴ cells) added to the cultures gave a negligible response to mitogens (less than 30 c.p.m.)

Table 2 3',5'-cyclic GMP-dependent release of lymphocyte-activating factor from peritoneal cells of mice

Source of supernatant	Stimulation index	
	Thymocytes	Spleen cells
PC + 3',5'-cyclic GMP	12.0 (23.2)	3.0
PC + 2',3'-cyclic GMP	0.9	1.1
PC + 3',5'-cyclic AMP	0.7	1.0
PC + 3',5'-cyclic GMP + 3',5'-cyclic AMP	11.0	2.8
PC + 3',5'-cyclic GMP + EGTA	13.0	2.8

Peritoneal cells (PC) of BALB/c mice were incubated in a culture medium (see Table 1) with either 3',5'-cyclic GMP (5 × 10⁻³ M), 2',3'-cyclic GMP (1 × 10⁻³ M), 3',5'-cyclic AMP (1 × 10⁻³ M), or a combination of 3',5'-cyclic GMP (5 × 10⁻³ M) and 3',5'-cyclic AMP (1 × 10⁻³ M), or a combination of 3',5'-cyclic GMP (5 × 10⁻³ M) and EGTA (0.6 mM) (see Table 1). Culture fluids were collected after 24 h and sterilised by filtration (Millipore filters). Thymocytes or spleen cells (2 × 10⁶ ml⁻¹) of BALB/c mice were then cultured as described in Table 1 in the presence of 10% various supernatants (final concentration). Incorporation of ³H-thymidine into the nuclear DNA was determined in 48 h cultures. Results are expressed as stimulation indices (c.p.m. in cultures in presence of 10% supernatant of PC cultures/c.p.m. in cells cultured without addition of supernatant). Each value represents mean of triplicate cultures, s.d. less than 10%. Value in brackets shows response of thymocytes to same supernatant after extensive dialysis against culture medium (without serum) for 24 h.

adherent cells. 3',5'-cyclic GMP induces the release of a soluble non-dialysable factor from peritoneal cells with lymphocyte (predominantly thymocyte)-activating properties. The factor seems to be similar or identical with the lymphocyte activating factor already described[7,9], indicating the possibility that the production of the factor is mediated by a 3',5'-cyclic GMP-dependent process.

This work was supported by the Deutsche Forschungsgemeinschaft (T.D.) and by the Graduiertenförderung der Freien Universität Berlin (A.U.).

TIBOR DIAMANTSTEIN
ARTUR ULMER

Immunological Research Unit,
Klinikum Steglitz, Freie Universität,
Hindenburgdamm 30, D-1 Berlin 45, Germany

1 Greaves, M. F., *et al.*, *Transplantn Rev.*, 11, 87 (1972).
2 Singer, S. J., *Adv. Immun.*, 19, 34 (1974).
3 Hadden, J. W., Hadden, E. M., Haddox, M. K., and Goldberg, N. D., *Proc. natn. Acad. Sci. U.S.A.*, 69, 3024 (1972).
4 Diamantstein, T., and Ulmer, A., *Immunology*, 28, 113 (1975).
5 Weinstein, Y., Chambers, D. A., Bourne, H. R., and Melmon, K. L., *Nature*, 251, 352 (1974).
6 Watson, J., *J. exp. Med.*, 141, 97 (1975).
7 Gery, I., and Waksman, B. H., *J. exp Med.*, 136, 143 (1972).
8 Diamantstein, T., and Ulmer, A., *Immun. Commun.* 4, 57 (1975).
9 Calderon, J., and Unanue, E R., *Nature*, 253, 359 (1975).
10 Goldberg, N D., O'Dea, R. F., and Haddox, M. K , *Adv. Cyclic Nucleotide Res.*, 3, 156 (1973).
11 Alford, R. H., *J Immun.*, 104, 698 (1970).
12 Diamantstein, T., and Ulmer, A., *Immunology*, 28, 121 (1975).
13 Diamanstein, T., Ruhl, H., Vogt, W., and Bochert, G., *Eur. J. Immun.*, 3, 488 (1973).

Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier

THE most exciting aspect of immunology at the moment is the way in which the major histocompatibility complex regulates certain functions of the immune system. Understanding the basis of this will revolutionise our thinking about T cell responsiveness and perhaps explain the puzzling degree of genetic polymorphism of the major histocompatibility complex. Most rewarding also will be the clinical applications, since susceptibility to many diseases is linked to HL-A (refs 1 and 2). Genes which control the response of thymus-derived (T) lymphocytes to certain antigens map in the major histocompatibility complex[1]; 1–10% of T lymphocytes respond in

an allogeneic mixed lymphocyte culture, or graft-*versus*-host reaction, against non-self major histocompatibility antigens[3-5]; cytotoxic T cells derived from mixed lymphocyte cultures seem to be directed against the classical serologically defined antigens coded in this region[6,7]. The work described here derives from another recently reported link between murine T cell function and the major histocompatibility complex. It has now been demonstrated that T-cell mediated lysis of virus infected target cells is restricted by H-2 (refs 8–10). Lysis of TNP modified
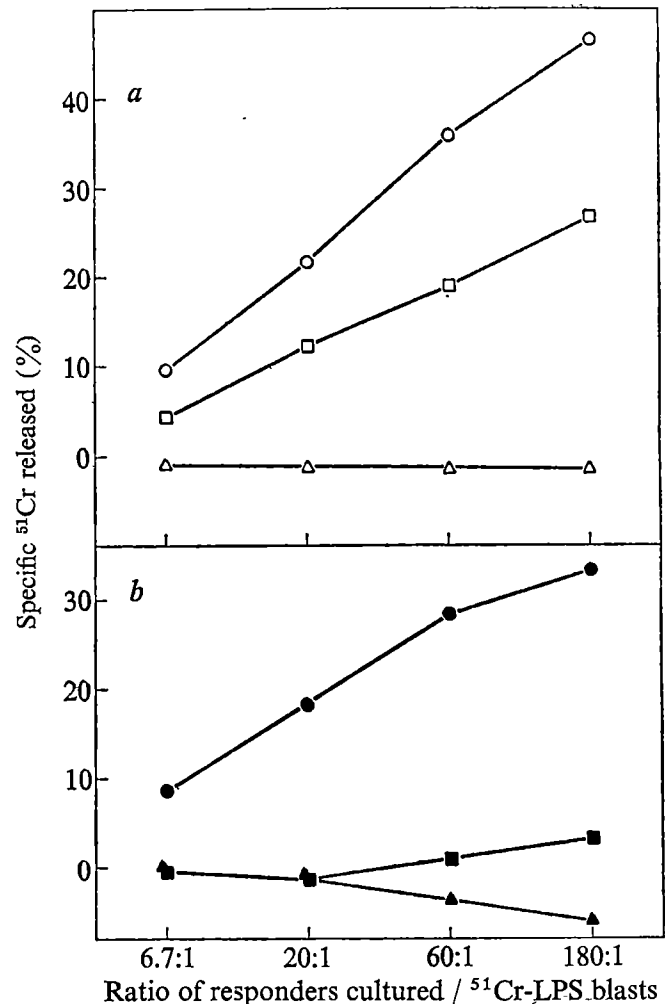
Fig. 1 Assay of lysis of six targets by BALB/c(H-2ᵈ) anti-B10.D2(H-2ᵈ) cytotoxic T cells. Normal BALB/c mice were immunised by intraperitoneal injection of 44 × 10⁶ spleen and lymph node cells from B10.D2 mice in Hanks' balanced salt solution. The spleen was removed 18 d later and 4 × 10⁶ viable cells ml⁻¹ were restimulated *in vitro* with 4 × 10⁶ cells ml⁻¹ irradiated (1,000 rad from a ⁶⁰Co source) B10.D2 spleen cells. Responder and stimulator cells were from female mice. After 4 d the cultures were collected and washed once before the assay. Targets were spleen cells which had been cultured for 3 d in the presence of 10 µg ml⁻¹ LPS to induce blast transformation of the B cells[7,13]. Targets were collected, washed, labelled with ⁵¹Cr-sodium chromate, washed twice, and viable blasts counted. Threefold dilutions of the responder cells were titred against a constant number (5 × 10⁴) of ⁵¹Cr-labelled LPS blasts for 4 h in 35-mm Petri dishes on a rocking platform. At the end of the assay, the contents of the dishes were transferred to tubes, centrifuged, and half the supernatant removed for counting. The results are plotted as the ratio of responders originally cultured to 5 × 10⁴ ⁵¹Cr-targets (logarithmic scale) against the percentage specific ⁵¹Cr-released (arithmetic scale) calculated as:

$$\frac{\text{Experimental release} - \text{spontaneous release}}{100 - \text{spontaneous release}} \times 100$$

Targets were: B10.D2(H-2ᵈ) (○); DBA/2(H-2ᵈ) (□); B10.BR (H-2ᵏ) (△); C57BL/6(H-2ᵇ) (■); B6.C/H-2ᵈ (synonym HW19) (●); and BALB/c(H-2ᵈ) (▲). Spontaneous release (release by 5 × 10⁴ targets cultured alone) varied from 18.2 to 25.9%.

targets is similarly restricted in another system[11]. I have now shown that cytotoxic T cells derived from mice immunised with cells from an allogeneic strain which carries the same H-2 region will lyse only targets which share the same H-2 as the immunising strain.

Schematically the results can be summarised as follows: cytotoxic T cells of strain A(H-2¹) immunised against cells of strain B(H-2¹) will lyse B(H-2¹) targets but not B(H-2²) targets; they do, however, crossreact strongly and kill cells of strains C(H-2¹) or D(H-2¹) but not C(H-2³) or D(H-2⁴). Such cytotoxic target antigens apparently include hybrid specific antigens (that is, antigens present on an F_1 but not on either homozygous parent). Schematically, A(H-2¹) anti-B(H-2¹) will not lyse A(H-2¹) nor B(H-2²) but will lyse F_1(A(H-2¹)×B(H-2²)) targets. I favour the hypothesis that all the allogeneic surface structures which are targets for cytotoxic cells in this system are interaction antigens created by an H-2 coded modification of the products of non-H-2 coded genes—probably minor histocompatibility genes.

The immunisations were done by priming the mice with an intraperitoneal injection of living allogeneic H-2 similar stimulating cells and 18–40 d later removing the spleen and boosting *in vitro* with irradiated stimulating cells. All the cytotoxic effector activity generated has been shown to be sensitive to treatment with anti-Thy-1.2 serum and complement immediately before the assay. Figure 1 shows the lytic activity of BALB/c (H-2ᵈ) anti-B10.D2 (H-2ᵈ) cells against targets from six strains of mice. Cells syngeneic with the responders were not lysed (Fig. 1*b*). B10.D2 was lysed very efficiently, but B10.BR (H-2ᵏ) which is genetically the same as B10.D2, except for the H-2 region, was not lysed (Fig. 1*a*). Targets from another unrelated strain, DBA/2, which carries H-2ᵈ were killed quite efficiently (Fig. 1*a*). A trivial explanation of this cross reaction was that, since B10.D2 derived its H-2ᵈ region from DBA/2, the BALB/c cells were immunised against a surface alloantigen closely linked genetically to, or within, the DBA/2 and B10.D2 H-2 region. This explanation is ruled out in Fig. 1*b*, which shows the results with another congenic pair of targets. C57BL/6 (H-2ᵇ) was lysed only very weakly by this population of BALB/c anti-B10.D2 cytotoxic cells, whereas B6.C/H-2ᵈ which is congenic with C57BL/6 and derived H-2ᵈ from BALB/c[12], was lysed very efficiently, about as well as B10.D2 targets.

Figure 2 shows the activity of BALB/c and B10.D2 cytotoxic cells obtained after immunisation with P815, a DBA/2 (H-2ᵈ) mastocytoma, when assayed at the same time against targets from six strains of mice. Neither population caused lysis of syngeneic targets. Both lysed cells of the immunising (DBA/2) strain very efficiently (Fig. 2*a* and *b*). BALB/c anti-P815 cross-reacted strongly on B10.D2, as would be predicted from Fig. 1,

but did not lyse B10.BR(H-2ᵏ) (Fig. 2*a*); B10.D2 anti-P815 on the other hand crossreacted strongly on BALB/c targets (Fig. 2*b*). The BALB/c cytotoxic population did not lyse syngeneic targets, and only caused slight lysis of C57BL/6 (H-2ᵇ), but F_1 (C57BL/6 × BALB/c) targets were lysed very well (Fig. 2*a*). Thus the cytotoxic T cells detected antigens found only on F_1 cells but not either parent, implying the existence of hybrid specific antigens.

In the experiment shown in Fig. 2*a*, BALB/c anti-DBA/2 cytotoxic cells lysed B10.D2 (H-2ᵈ) targets efficiently but not B10.BR(H-2ᵏ) targets, even though the two strains are congenic and differ only at H-2. It seemed possible that B10.BR did express antigens which the cytotoxic cells recognised on B10.D2, but the lytic function was somehow impaired by the different H-2 antigens. The question was thus whether the lysis of ⁵¹Cr-labelled B10.D2 targets caused by BALB/c anti-DBA/2 cytotoxic cells could be inhibited by the addition of unlabelled B10.BR cells. In this "cold target competition" experiment, damage of B10.BR cells was not measured, only their capacity to be bound by receptors on the cytotoxic cell[7,14]. As a control, unlabelled targets of BALB/c (syngeneic with the cytotoxic cells) or B10.D2 (syngeneic with the labelled target) were used as inhibitors. Table 1 shows that an 8 or 33-fold excess of B10.BR cells over ⁵¹Cr-B10.D2 cells did not inhibit release of label any more than did the same excess of BALB/c cells. Unlabelled B10.D2 naturally inhibited lysis. This is strong evidence that the cytotoxic cells did not see the same non-H-2 coded antigens on B10.BR as they did on B10.D2.

Two hypotheses will now be considered to explain this finding that, even after an allogeneic immunisation when responder and stimulator share the same H-2, there is a requirement in lysis for the target also to share the same H-2. The first is that there is dual recognition by the cytotoxic effector cell of H-2 coded and non-H-2 coded structures on the target cell surface. This could mean that the cytotoxic cell receptor for non-H-2 is clonally restricted but all cells bear receptors for H-2, that is, a requirement for self-H-2 recognition. There are four lines of evidence against this. First, Zinkernagel and Doherty showed that F_1 cells immune to LCM-infected F_1 cells, after boosting in parent 1 virus-infected hosts, killed infected cells of parent 1 but not of parent 2 (ref. 15). Second, I have performed a similar experiment in which F_1 (BALB/c × BALB.B) (H-2ᵈ/ᵇ) immune to B10.D2 (H-2ᵈ) did lyse B10.D2, but did not lyse B10 (H-2ᵇ). Third, the cold target competition experiment reported here (Table 1) shows that B10.BR cells fail to inhibit the binding of BALB/c anti-DBA/2 cytotoxic T cells to B10.D2 targets. Fourth, cytotoxic cells with specificity for foreign H-2 exist, with no apparent requirement in lysis for recognition of other surface structures. Alternatively, both the cytotoxic cell re-

Table 1 Inhibition by unlabelled cells of the lysis of ⁵¹Cr-B10.D2 targets mediated by BALB/c anti-DBA/2 cytotoxic cells

Unlabelled LPS blasts used for inhibition*	Genotype: H-2	background	Experimental–spontaneous release†	Factor of inhibition of lysis‡
None	—	—	37.7	—
BALB/c (×8)	d	BALB/c	32.4	1.0
BALB/c (×33)	d	.BALB/c	29.3	1.0
B10.BR (×8)	k	B10	32.3	1.0
B10.BR (×33)	k	B10	30.9	0.9
B10.D2 (×8)	d	B10	17.7	3.2
B10.D2 (×33)	d	B10	6 3	13.6

BALB/c mice were immunised by intraperitoneal injection of 10⁷ P815 and by a secondary boost *in vitro* with 5,000 rad irradiated P815. These cytotoxic BALB/c anti-DBA/2 cells were titred against 5 × 10⁴ ⁵¹Cr-labelled B10.D2 LPS blasts for 4 h at ratios of from 43:1 to 0.18:1. At a ratio of 12:1, an 8-fold (4 × 10⁵), or 33-fold (1.65 × 10⁶) excess of unlabelled LPS blasts from BALB/c, B10.BR or B10.D2 were added to the assay to inhibit ⁵¹Cr release

*Numbers in parentheses refer to the numerical excess of unlabelled LPS blasts over ⁵¹Cr-B10.D2 blasts present in the assay.
†Spontaneous release was 34.8%.
‡A factor of 1.0 refers to no inhibition relative to the same excess of BALB/c LPS blasts. An 8-fold excess of BALB/c blasts in fact gave a 1.8-fold inhibition, and a 33-fold excess a 2.4-fold inhibition compared with when no unlabelled inhibitor cells were present. The factor of inhibition was calculated from the titration curve as described previously[7].
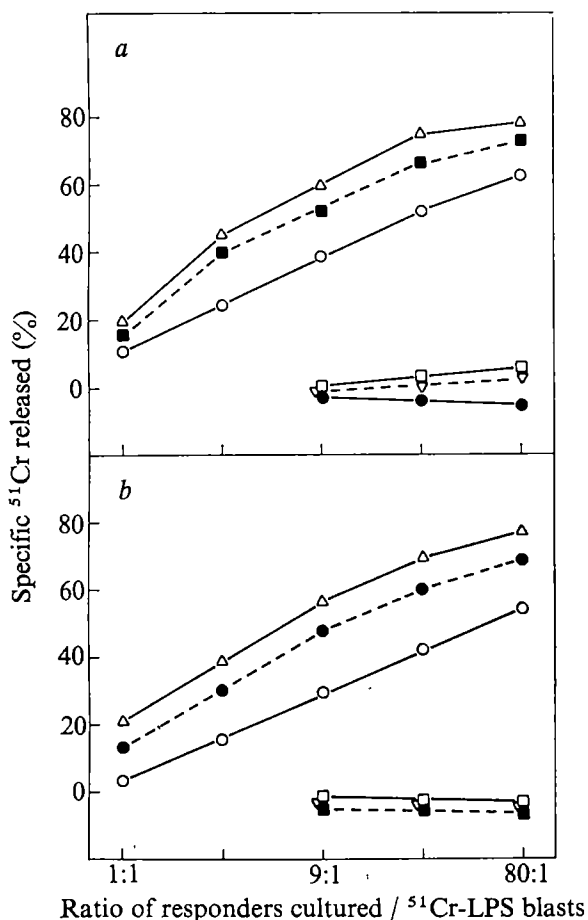
Fig. 2 Lysis of six targets by cytotoxic T cells from: *a*, BALB/c (H-2^d) and *b*, B10 D2(H-2^d) mice immunised against DBA/2 (H-2^d) cells. Responder mice were injected intraperitoneally with 10^7 viable DBA/2 mastocytoma cells, P815, which were grown in tissue culture. 20 days later the spleen was removed and cultured at 4×10^6 cells ml^{-1} with 2×10^5 cells ml^{-1} irradiated (5,000 rad from a ^{60}Co source) P815. After 4 d the cells were washed and threefold dilutions titred against ^{51}Cr-labelled LPS blast targets as in Fig. 1 Targets were: DBA/2(H-2^d) (△); B10.D2(H-2^d) (■); C57BL/6(H-2^b) (□); B10.BR(H-2^k) (▽); BALB/c(H-2^d) (●); and F$_1$(C57BL/6 × BALB/c) (○). Spontaneous release varied from 24.2 to 31.4%.

ceptor for non-H-2 and for H-2 may be clonally restricted. Evidence against this includes the third and fourth arguments above.

Hypothesis 1 is rejected at this stage of experimentation in favour of the second. This states that the H-2 region codes for a product which interacts with the products of genes coded elsewhere, and the result of this is the creation of new complex determinants (interaction antigens) which are recognised by allogeneic cytotoxic T cells. Although viral infection may alter H-2 antigens[16], the targets in my work are normal cells and a wealth of previous literature[6,17] and one preliminary experiment performed in this laboratory do not suggest a difference in the H-2 antigens on BALB/c against those on DBA/2 or B10.D2 which are recognised by anti-H-2^d cytotoxic cells. I therefore favour the explanation that H-2 codes for a modifier of all the other surface components which are targets in this system. In a hybrid, such as F$_1$ (C57BL/6 (H-2^b) × BALB/c (H-2^d)), the maternal (C57BL/6) and paternal (BALB/c) non-H-2 coded components are modified by both the maternal (H-2^b) and paternal (H-2^d) H-2 complex. This results in the creation of hybrid specific alloantigens, that is, the H-2^d (paternal) modification of the C57BL/6 (maternal) non-H-2 coded components and the H-2^b (maternal) modification of the BALB/c (paternal) non-H-2 coded components (Fig. 2a). The strong cross reaction shown here between unrelated strains with the same H-2 haplotype can be explained if the non-H-2 components are coded by minor

histocompatibility genes. Strains differ by a large number of minor histocompatibility loci but the number of alleles at each locus is small[18]. The basis for the cross reaction between DBA/2 and BALB/c detected by B10.D2 (Fig. 2b) is explicable if the two strains share the same allele at some minor histocompatibility loci where B10.D2 carries a different allele.

In terms of the molecular nature of the H-2 coded modification, the most attractive idea at the moment is that the major histocompatibility complex codes for a glycosyl transferase system which uses as substrate the products of genes which code for surface components (P. Trefts and B. E. Rothenberg, unpublished). This predicts that in allogeneic T-cell mediated killing the target antigens are carbohydrate in nature.

I thank Melvin Cohn, Rod Langman, Park Trefts and Barry Rothenberg for discussion. This work was supported by the US National Institute of Allergy and Infectious Diseases.

MICHAEL JOHN BEVAN

The Salk Institute for Biological Studies,
PO Box 1809,
San Diego, California 92112

1 McDevitt, H. O , and Landy, M., (eds) *Genetic Control of Immune Responsiveness: Relationship to Disease Susceptibility*, (Academic, New York, 1972).
2 Morris, P. J., *Contemporary Topics in Immunobiology* (edit. by Cooper, M. D., and Warner, N L.), 3, 141–169 (Plenum, New York, 1974).
3 Wilson, D. B., Blyth, J L., and Nowell, P. C., *J. exp. Med.*, 128, 1157–1181 (1968)
4 Atkins, R C., and Ford, W. L., *J. exp Med.*, 141, 664–680 (1975).
5 Ford, W L , Simmonds, S J., and Atkins, R. C., *ibid.* 681–696.
6 Nabholz, M., Vives, J , Young, H. M , Meo, T , Miggiano, V., Rinjbeck, A., and Shreffler, D. C , *Eur J Immun* , 4, 378–387 (1974)
7 Bevan, M J , *J. Immun* , 114, 316–319 (1975).
8 Zinkernagel, R. M , and Doherty, P. C., *Nature*, 248, 701–702 (1974).
9 Doherty, P C., and Zinkernagel, R M., *J. exp Med.*, 141, 502–507 (1975)
10 Blanden, R V., in *Progress in Immunology*, 2 (edit by Brent, L , and Holborow, J) 4, 117–125 (1974).
11 Shearer, G. M., *Eur. J. Immunol* , 4, 527–533 (1974).
12 Klein, J., *Transplantation*, 15, 137–153 (1973)
13 Bevan, M J., and Cohn, M., *J. Immun.*, 114, 559–565 (1975)
14 Ortiz de Landazuri, M., and Herberman, R. B., *Nature new Biol.*, 238, 18–19, (1972).
15 Zinkernagel, R M., and Doherty, P. C., *Nature*, 251, 547–548 (1974).
16 Gardner, I D , Bowern, N. A., and Blanden, R. V., *Eur. J Immunol.*, 5, 122–127 (1975).
17 Cerottini, J. C., and Brunner, K. T , *Adv. Immun.*, 18, 67–132 (1974)
18 Graff, R. J , and Bailey, D W., *Transplant Rev.*, 15, 26–49 (1973)

Interchange of adenyl and guanyl cyclases as an explanation for transformation of β- to α-adrenergic responses in the rat atrium

STUDIES have indicated that the response of the heart[1-5], and possibly other tissues[6], to exogenous catecholamines changes from β-type to α-type in conditions of decreased metabolic activity, including lowering the temperature. Attempts to correlate this with changes in cyclic AMP metabolism which are thought to reflect β-adrenoceptor activity were unsuccessful[7,8]. We have therefore studied the effects of lowering the temperature on the intracellular levels of both cyclic AMP and cyclic GMP in the spontaneously beating rat atrium. Cyclic GMP concentrations seem to mediate α-adrenergic responses in a variety of systems[9-12], and we therefore investigated whether its levels increased in conditions of increased α-adrenoceptor activity. Our results indicate that the same receptors that stimulate cyclic AMP synthesis at 37 °C, trigger the synthesis of cyclic GMP at a colder temperature (24 °C).

Figure 1 shows that the changes in cyclic nucleotide concentration induced by the low optimal concentrations of three catecholamines preceded their chrono- and inotropic effects. Increases in cyclic GMP levels were more prominent at 24 °C than at 37 °C, whereas changes in cyclic AMP were not impressive and confirm the previously observed lack of correlation between cyclic AMP levels and β-adrenoceptor activity at low agonist concentrations[13].

When peak changes in rate × force were used as an index of atrial performance, lowering the temperature to 24 °C resulted in transformation of β to α-adrenoceptors (Fig. 2).
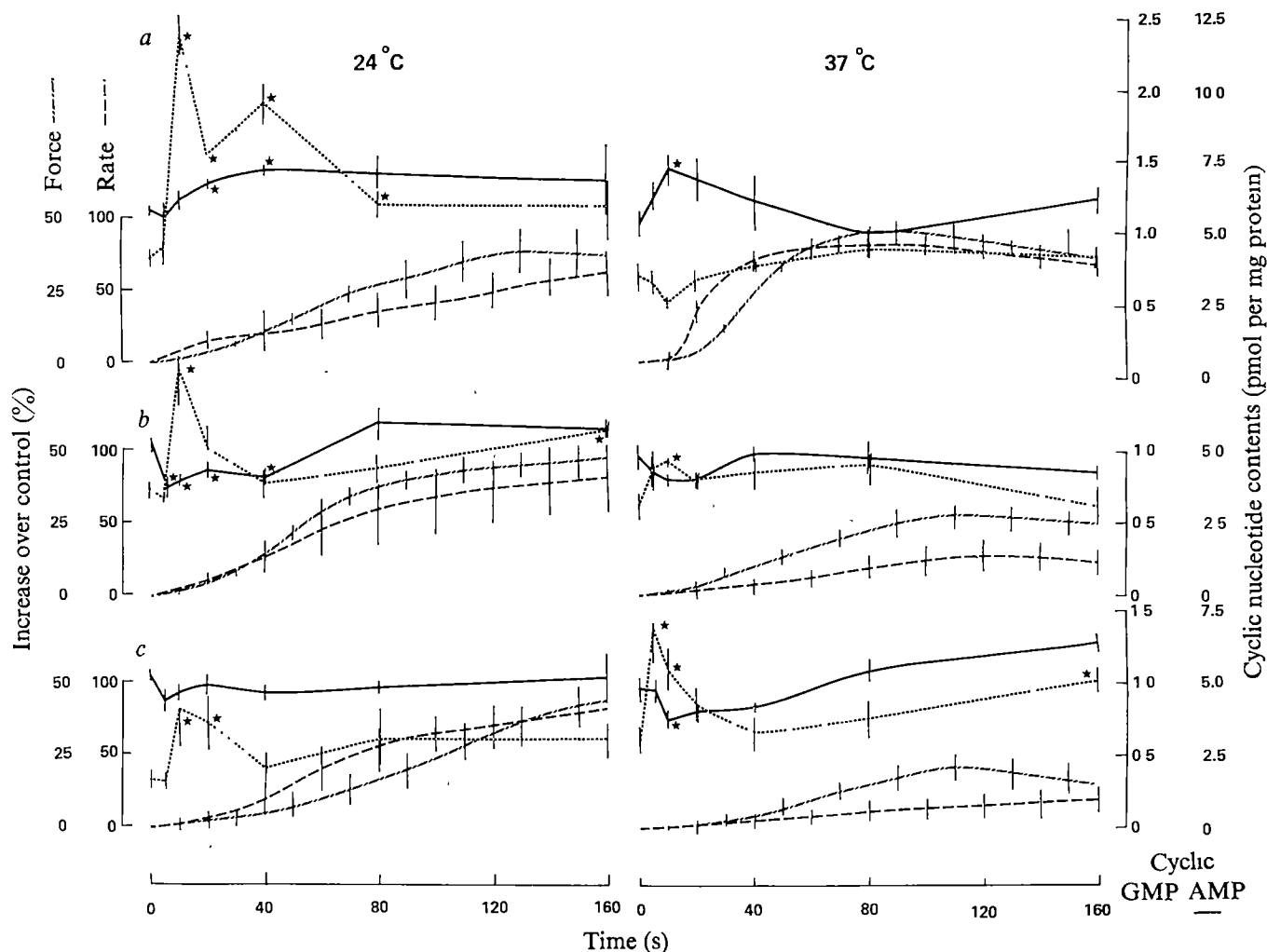
Fig. 1 Time course of effect of three catecholamines on rat atrial performance and cyclic nucleotide contents at 24 and 37 °C. a, Isoproterenol; b, adrenaline; c, noradrenaline Right and left atria were removed together from 150–200 g male Sprague–Dawley rats and suspended in a tissue bath containing 25 ml Chenoweth's solution[14] aerated with 5% CO_2 in O_2. Bath temperature was maintained at 24 or 37 °C throughout. Right atrial appendage was fixed in the bath, left atrial appendage was attached by a fine silk thread to a Grass FT03 force transducer, and contractile activity was recorded with a Grass model 7 polygraph. Diastolic tension was maintained at 2 g. Atria were allowed to equilibrate for 30 min Spontaneous heart rate and contractile force were obtained from the record and were 98–125 beats min^{-1} and 0 82 to 0.97 g at 24 °C and 286–304 beats min^{-1} and 0.28 to 0 37 g at 37 °C, respectively, immediately before the additions of the agonists (10^{-7} M) which were added to the bath at zero time. To determine cyclic nucleotide levels, atria were quickly frozen at the predetermined times using modified Wollenberger clamps previously cooled in liquid nitrogen Atria were kept frozen at −70 °C until analysed for cyclic nucleotide contents not more than 2 weeks later The two cyclic nucleotides were separated from each other on Dowex–formate columns[15]. Cyclic AMP was determined by the protein binding method of Gilman[16] and cyclic GMP was determined by radioimmunoassay[17]. Internal standards of tritiated cyclic nucleotides were used to calculate recoveries, which were 87–95% for cyclic AMP and 55–78% for cyclic GMP. Phosphodiesterase treatment destroyed all assayable cyclic nucleotides. Data for both atrial performance and cyclic nucleotide contents are the average of six experiments ±s.e.m. ─ ─ ─, Percentage change in rate, ─ ─ ─, percentage change in force, ─────, cyclic AMP contents; · · · · ., cyclic GMP contents. *Statistical significance from corresponding zero time control.

This is shown by the disappearance of the order of potency, isoproterenol > adrenaline or nonadrenaline, characteristic of the β-receptor; and the greater susceptibility of the catecholamine effects to blockade by the α-adrenoceptor blocker phentolamine, which is ineffective at 37 °C; at this temperature propranolol or sotalol (not shown) were the more effective blockers. Similar results were obtained when rate alone instead of rate × force was used as an index of atrial function, except that the effects of noradrenaline in the cold were less susceptible to blockade by phentolamine. These data agree well with those reported for frog and rat hearts[1-4].

At 37 °C cyclic AMP levels seemed to reflect β-adrenoceptor activity, as isoproterenol was more effective than adrenaline and noradrenaline in raising this cyclic nucleotide level, particularly at the higher agonist concentration (Table 1). In addition the changes in cyclic AMP induced by isoproterenol and adrenaline were blocked by the β-adrenergic blocker propranolol, thus emphasising the β-adrenoceptor nature of the cyclic AMP synthesising system. When the temperature was lowered to 24 °C, decreased effectiveness of isoproterenol and increased

activity of both adrenaline and noradrenaline in promoting cyclic AMP synthesis were observed. Furthermore, increases in cyclic AMP levels by isoproterenol and adrenaline were susceptible to blockade by the α-adrenoceptor blocker phentolamine.

At 37 °C cyclic GMP synthesis seemed to reflect the activity of the α-adrenoceptor as the order of potency of the catecholamines used was noradrenaline > adrenaline > isoproterenol (see also Fig. 1); and the effects, particularly at the low agonist concentrations, were susceptible to blockade by the α-adrenoceptor blocker phentolamine (see also Fig. 2). When the atria were cooled to 24 °C however, the reverse order of potency of the three catecholamines on cyclic GMP synthesis became apparent although the effects were again susceptible to blockade by phentolamine. In addition, the β-adrenergic blocking agent propranolol effectively blocked the cyclic GMP increases induced by all catecholamines at the colder temperature

It seems therefore that the same set of β-receptors with its classical structural requirements and blocker susceptibility that trigger the synthesis of cyclic AMP at 37 °C, promoted the

Table 1 Effects of various concentrations of agonists alone and in the presence of antagonists on cyclic nucleotide levels in the rat atrium at 24 and 37 °C

| | | Cyclic AMP content (pmol per mg protein) | | | | | | Cyclic GMP content (pmol per mg protein) | | | | | |
| | | 24 °C | | | 37 °C | | | 24 °C | | | 37 °C | | |
		Alone	+Pro-pranolol	+Phen-tolamine	Alone	+Pro-pranolol	+Phen-tolamine	Alone	+Pro-pranolol	+Phen-tolamine	Alone	+Pro-pranolol	+Phen-tolamine
Isoproterenol	10⁻⁷M	5.7 ± 0.4	10.1 ± 1.1†	9.5 ± 0 6†	6.7 ± 0.7	4.5 ± 0 6‡	6.6 ± 0.3	2 30 ± 0 18	0 31 ± 0.01†	0.82 ± 0.32*	0.42 ± 0 03	0.23 ± 0.02*	0 36 ± 0.08
	10⁻⁶M	18.3 ± 1.5†	—	—	19.6 ± 1 0†	—	—	0.65 ± 0 10*	—	—	0.50 ± 0 14	—	—
	10⁻⁵M	22.3 ± 2 8†	12.9 ± 0 8†	15.6 ± 0.6‡	37 1 ± 8.3*	13.4 ± 0 2‡	20 7 ± 1.2	0.57 ± 0.07*	0 48 ± 0 02	1.19 ± 0 12‡	0 74 ± 0 04*	0.36 ± 0 08‡	1.11 ± 0.19
Adrenaline	10⁻⁷M	4.4 ± 0 3	8.9 ± 0.7†	9.3 ± 0.5†	4 0 ± 0.2	4.7 ± 0 6	7 8 ± 0.5†	1.34 ± 0 18	0.40 ± 0.10†	0.45 ± 0.05*	0 93 ± 0 02	0 26 ± 0 03*	0.59 ± 0.03*
	10⁻⁶M	14 1 ± 0.4†	—	—	10.8 ± 1 3†	—	—	0 52 ± 0.06*	—	—	0.16 ± 0.05*	—	—
	10⁻⁵M	16.8 ± 1.5†	11.9 ± 0.2§	12.5 ± 0.6†	15.3 ± 0.4†	9 9 ± 0.5§	12 5 ± 0.3§	0.46 ± 0 03*	1 03 ± 0 16‡	0.63 ± 0 11	0.44 ± 0.03*	0 48 ± 0 06	1.10 ± 0.23
Noradrenaline	10⁻⁷M	5.2 ± 0.3	10.2 ± 1.6*	9.0 ± 1.0†	3.3 ± 0.3	4.5 ± 0 5	8.9 ± 0 5†	0.92 ± 0.21	0 66 ± 0.12	0.25 ± 0.03*	1 09 ± 0 14	0 36 ± 0 05*	0.75 ± 0.10*
	10⁻⁶M	13.4 ± 0.6†	—	—	8.7 ± 0.6†	—	—	0.39 ± 0.09	—	—	0.31 ± 0.06†	—	—
	10⁻⁵M	17.3 ± 1.1†	13.7 ± 0.6‡	17.5 ± 1.0	11.8 ± 0 9†	12 8 ± 1.0	14.0 ± 1 2	0.63 ± 0 05	0.42 ± 0.06	0 53 ± 0.07	0.20 ± 0.06*	0.85 ± 0 25†	0.73 ± 0 18‡

Experimental conditions and antagonist concentrations are the same as for Fig 2. Results are means of six experiments ± s.e.m.
*Statistical significance from agonist alone at 10⁻⁷M, P 0.05, †$P>0$ 01.
‡Statistical significance from agonist alone at 10⁻⁵M, $P<0.05$. §$P>0$ 01

synthesis of cyclic GMP at the colder temperature. Increased cyclic GMP synthesis at the colder temperature could not have resulted from the activation of new α-adrenoceptors that were dormant at 37 °C, as the order of potency noradrenaline > adrenaline > isoproterenol on cyclic GMP generation characteristic of classical α-adrenoceptors did not prevail. Increased cyclic GMP synthesis triggered by the β-adrenoceptor at the colder temperature may be regarded as either a change in substrate specificity of the catalytic subunit of the adenyl cyclase enzyme from ATP to GTP with no change in the discriminator or receptor subunit[18]; or the coupling of the β-adrenoceptor subunit at the cold temperature with the catalytic subunit of guanyl cyclase. A model conforming with

this latter possibility has already been proposed[19]. The continued susceptibility of cyclic GMP synthesis at the low temperature to the blocking effects of phentolamine indicates that this blocker must act as a cyclic GMP-generating site located past the apparently unchanged receptor, as has already been suggested[20]. Propranolol, on the other hand, seems to act at the level of the receptor subunit itself.

At the colder temperature and low agonist concentrations, the observed propranolol-resistant increases in cyclic AMP in the presence of the β-adrenergic blocking agent could be secondary to blockade of the increases in cyclic GMP (Fig. 2). This may also explain cyclic AMP increases in the presence of phentolamine at both 37 and 24 °C, as both cyclic nucleotides
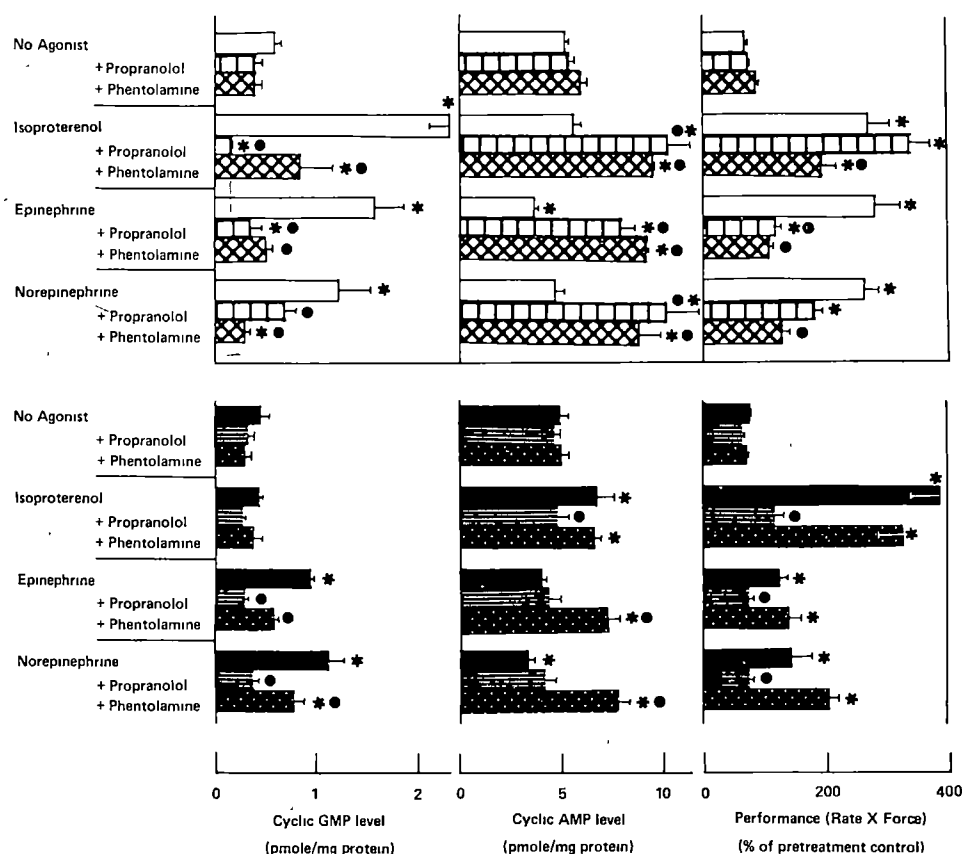
Fig. 2 Effects of various adrenergic agonists and antagonists on performance, cyclic AMP and cyclic GMP levels in spontaneously beating rat atria at 37 °C (*a*) and 24 °C (*b*). Experimental conditions are the same as for Fig. 1. Column heights represent the averages of 4–6 atria ±s.e m. *Statistically significant differences ($P<0.05$) from control (no agonist) at the same temperature; ● statistically different from agonist alone at the same temperature. Isoproterenol, adrenaline, noradrenaline and propranolol were added in a final concentration of 10⁻⁷ M whereas the final concentration of phentolamine was 10⁻⁶ M. Propranolol and phentolamine were added to the bath 30 min before and the atria were quickly frozen 10 s after addition of catecholamines. Mean (±s.e.) heart rates during the control (predrug) period were 292±6.4 and 105±8.5 beats min⁻¹ at 37 and 24 °C, respectively. Basal contractile forces were 0.29±0.03 g at 37 °C and 0.79±0.08 g at 24 °C.

are known to influence the rate of hydrolysis of one another[18]. The reverse situation may exist in the case of propranolol at the higher temperature, that is, blockade of the changes in cyclic AMP levels induced by the catecholamines may be responsible for the effects of propranolol on cyclic GMP levels. It is unlikely that the effects of these catecholamines on cyclic nucleotide levels involve direct action on phosphodiesterase enzymes[21]. It is not clear from the present study which cyclic nucleotide has the major role of translating the effects of catecholamines into mechanical effects in the heart, particularly in the crucial low temperature–low concentration conditions. The data presented here indicate a possible antagonistic role for cyclic GMP to that of cyclic AMP, a role that seems less prominent at the lower temperature. This is supported by the observation that cyclic GMP seems to mediate the negative chrono- and inotropic effects of acetylcholine on the heart[19].

M. Samir Amer
Jeffrey E. Byrne

*Department of Pharmacology,
Mead Johnson Research Center,
Evansville, Indiana 47721*

1 Kunos, G., and Szentivanyi, M , *Nature*, 217, 1077–1078 (1968).
2 Buckley, G A , and Jordan, C C , *Br J Pharmac* , 38, 394–398 (1970)
3 Kunos, G., Yong, M S., and Nickerson, M., *Nature new Biol* , 241, 119–120 (1974).
4 Hari, M. N. E., *Acta Pharmac. Toxic.*, 33, 273–279 (1973)
5 Kunos, G , Vermes-Kunos, I , and Nickerson, M , *Nature*, 250, 779 (1974)
6 Matheny, J L., and Ahlquist, R P , *Archs int. Pharmacodyn* , 209, 197–203 (1974)
7 Caron, M. G., and Lefkowitz, R J , *Nature*, 249, 258–260 (1974)
8 Benfey, B G , Kunos, G , and Nickerson, M , *Br J Pharmac* , 51, 253–257 (1974)
9 Schultz, G , Hardman, J. G , Schultz, K , Davis, J W , and Sutherland, E. W , *Proc natn Acad Sci U S A* , 70, 1721–1725 (1973)
10 Amer, M S., *Gastroenterology*, 67, 333–337 (1974).
11 Glass, D. B., White, J G , and Goldberg, N D , *Fedn Proc*., 33, 611 (1974)
12 Ball, J. H , *et al* , *J. clin Invest.*, 51, 2124–2132 (1972)
13 Sobel, B E., and Mayer, S E., *Circulation Res* , 32, 407–414 (1973)
14 Chenoweth, M B , and Koelle, E S , *J. Lab clin. Med* , 31, 600–608 (1946)
15 Murad, F., Manganiello, V , and Vaughan, M , *Proc. natn. Acad Sci U S A.*, 68, 736–739 (1971)
16 Gilman, A. G , *Proc. natn Acad. Sci U.S.A.*, 67, 305–309 (1970)
17 Steiner, A L , Parker, C W , and Kipnis, D M , *J biol Chem* , 247, 1106–1113 (1972)
18 Amer, M S , and McKinney, G R , *A Rep med Chem* , 9, 203–212 (1974).
19 Cuatrecasas, P , *Biochem Pharmac* , 23, 2353–2361 (1974)
20 Moran, N C., *Frontiers in Catecholamine Research*, 291–294 (Pergamon, Oxford, 1973)
21 Amer, M. S., and Kreighbaum, W E , *J Pharm Sci* , 64, 1–37 (1975)
22 George, W. J , Wilkerson, R. D., and Kadowitz, P J , *J. Pharmac exp. Ther* , 184, 228–235 (1973)

Two mechanisms for poststimulus hyper-polarisations in cultured mammalian neurones

Long lasting hyperpolarising responses following high frequency spike discharges in neurones from several sub-mammalian species have been attributed to either electrogenic ion transport[1,2] or to a long lasting increase in K^+ conductance[3,4]. These prolonged hyperpolarising responses of whatever mechanism result in alterations of neuronal behaviour which may have important functional consequences for the integrative activity of the central nervous system (CNS). Similar potentials have been reported in studies on the intact mammalian CNS[5–7], but the complexity of these preparations has prevented a thorough analysis. Such phenomena can be studied using intracellular recording techniques in dissociated cell cultures of mammalian neural tissue, and here we demonstrate post-stimulus hyperpolarising responses in cultured spinal neurones and suggest the underlying mechanisms.

Spinal cords were dissected from 13-d-old mouse embryos, mechanically dissociated and plated at a density of about 10^6 cells per dish (35 mm diameter, Falcon). After a brief exposure to a metabolic inhibitor (to suppress the rapid division of fibroblasts) cells were allowed to mature for a period of about 3 weeks. Cells were maintained in modified Eagle's medium with 10% horse serum added as described previously[8]. Experiments were carried out by placing a culture dish in a chamber on the modified stage of an inverted phase contrast microscope. Mature cultures contained two easily distinguishable populations of large neurones (25–50 µm): dorsal root ganglion (DRG)

cells and multipolar spinal cord (SC) cells. These cells exhibited a full range of characteristic neuronal electrophysiology including membrane potentials of -40 to -60 mV, spike electrogenesis, and in the case of SC cells, synaptic transmission, as well as sensitivity to a number of putative transmitters[8,9]. Neuronal somata were impaled under direct vision with 4 M K^+ acetate-filled pipettes (20–40 MΩ). At the time of study $MgSO_4$ was usually added to a final concentration of 10 mM to suppress spontaneous synaptic activity. Standard electrophysiological techniques were used for intracellular recording and iontophoresis. Glutamate (0.6 M, *p*H 8) was iontophoresed on to cell somata from fine-tipped pipettes. Strophanthidin (3×10^{-4}– 7×10^{-4} M, Sigma), dissolved in medium, was leaked on to cells using coarse pipettes (tip diameter 25–50 µm).

Glutamate, a putative excitatory transmitter in the mammalian CNS[10], caused brisk depolarisation, usually evoking action potentials in over 95% of SC cells. Short pulses of glutamate (that is < 1 s duration) elicited responses with roughly exponential falling phases. Longer lasting pulses evoked a more complicated sequence of events (Fig. 1*a*). During the drug application the cells depolarised to a steady plateau level without evidence of desensitisation. Following termination of the iontophoretic current, the membrane potential returned past the prestimulus level resulting in a long lasting (20–200 s) hyperpolarisation of variable magnitude (1–20 mV). The magnitude and duration were related directly to the duration and intensity of the glutamate application. This post-glutamate hyperpolarisation (PGH) was not associated with a change in input resistance (Fig. 1*b*) and did not have an apparent reversal potential. Conditioning hyperpolarisation of the membrane potential (through sustained injection of current) enhanced the PGH response in seven cells tested (Fig. 1*c*). PGH was present at potentials at which the hyperpolarising after-potential of the spike had reversed polarity, implying that the PGH could drive the membrane potential more negative than the K^+ equilibrium potential.

PGH was rapidly and reversibly abolished by the local application of strophanthidin in six cells tested (Fig. 1*b*). In four cells application of the drug during PGH caused an

Fig. 1 Characteristics of PGH. *a*, Response of multipolar spinal cord cell to prolonged glutamate application (50 nA, shown by bar). Trace 2 is a high gain record of trace 1. Membrane potential -38 mV. *b*, Effect of strophanthidin on PGH. Constant applications of glutamate (34 nA, 8 s) were given before (con), during (stroph), and 5 min after (post) the local application of strophanthidin (3×10^{-4} M) to the cell soma Downward deflections are voltage responses to injection of constant hyperpolarising current pulses (0.3 nA for con and stroph, 0.5 nA for post record) Input resistance does not change during PGH The post PGH is longer lasting and smaller than the con PGH probably as a result of a reduction in peak pump rate caused by residual strophanthidin action. Membrane potential -65 mV. *c*, Relationship between PGH amplitude and membrane potential Constant glutamate applications (50 nA, 12 s) were applied while the membrane potential was varied by passing steady polarising current through the recording electrode Membrane potential at rest -38 mV.
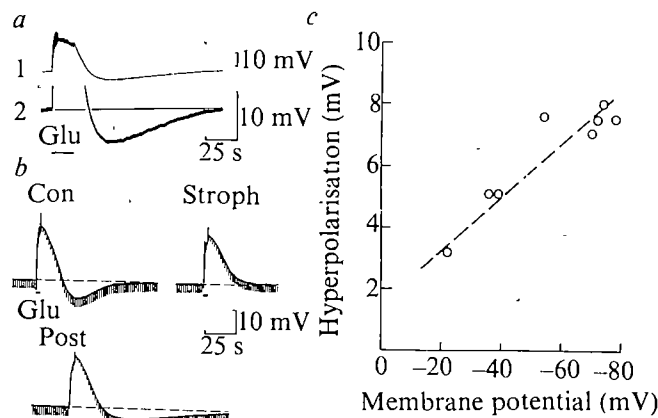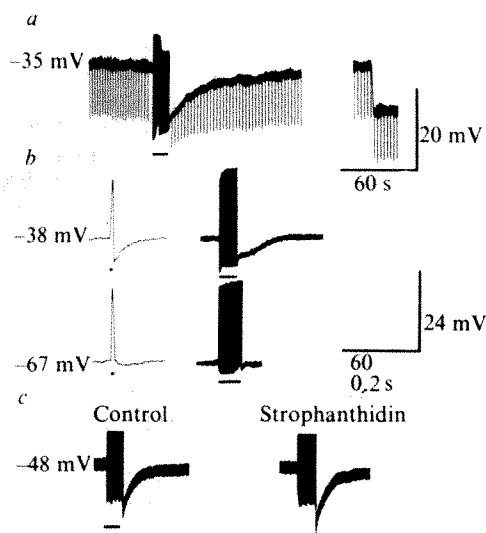
Fig. 2 Characteristics of PTH in DRG cells. a, Trains of depolarising current pulses (30 s⁻¹) elicited a spike train of same frequency (shown by bar), following which a slowly relaxing hyperpolarisation is seen. Downward deflections are voltage responses to the injection of constant current pulses (0.2 nA). Note the decrease in input resistance during PTH. Right-hand record in a shows that no change in resistance occurs when the membrane potential of this cell is passively moved in the hyperpolarising direction by steady current injection. b, Relationship between spike after-potential, PTH and membrane potential. After-potential following an action potential (induced by a single depolarising current pulse) and PTH elicited in the same cell by high frequency pulses (50 s⁻¹) are compared at different membrane potentials achieved by polarising current. c, Locally applied strophanthidin (7 × 10⁻⁴ M) does not alter PTH (50 s⁻¹).
Calibration: 20 mV and 60 s (a and c); 0.2 s (b, left).

immediate depolarisation averaging 15 mV. (Strophanthidin application to cells not undergoing PGH led to rapid, reversible, depolarisation averaging 5 ± 2 mV (s.d.; $n = 12$).) Drug application did not cause any detectable changes in input resistance, nor did it interfere with the depolarisation elicited by glutamate.

DRG cells, which are unresponsive to glutamate (B.R.R. and P.G.N., unpublished), also exhibited a form of poststimulus hyperpolarisation. In these cells high frequency spike trains (elicited by depolarising current pulses) were followed by hyperpolarisations lasting several seconds (Fig. 2a). Although this post-tetanic hyperpolarisation (PTH) is similar to the hyperpolarising responses in SC cells with regard to magnitude and duration, PTH has several distinguishing characteristics. During PTH, input resistance was decreased, returning to its control value as the membrane potential returned to the baseline level (Fig. 2a). PTH magnitude was dependent on membrane potential; its reversal potential was identical to that of the spike after-potential (Fig. 2b). Strophanthidin, applied to DRG cells, did not alter PTH (Fig. 2c).

PTH in DRG cells seems to result from a prolonged period of increased K⁺ conductance. This is suggested by the conductance increase during PTH, the reversal potential of PTH being coincident with the reversal potential of the spike afterpotential, and the insensitivity of PTH to strophanthidin. In invertebrate cells a response with similar characteristics follows spike trains and has been shown to depend on Ca²⁺ influx during action potentials with a subsequent increase in K⁺ conductance [3,4]. As the DRG cell has a Ca²⁺ component to its spike (J. Fischbach and M. Dichter, unpublished), it is possible that PTH may result from a similar sequence of events.

PGH in SC cells fulfils many of the electrophysiological criteria which identify potentials produced by electrogenic transport[2]: it occurs without change in membrane conductance, can exceed the K⁺ equilibrium potential (as reflected by the reversal potential of the spike after-hyperpolarisation), and is abolished by the glycoside strophanthidin at a concentration known to inhibit the Na⁺ pump. As the glutamate response has

an equilibrium potential around −20 mV, it is likely that the response involves a substantial increase in Na⁺ permeability (ref. 11 and N. Brookes, unpublished). Prolonged glutamate application therefore would lead to an increase in intracellular Na⁺ concentration, causing an increase in the rate of Na⁺ pumping[12]. In fact, spike amplitude is depressed during PGH, suggesting that the Na⁺ gradient across the membrane is reduced (not illustrated). Other experiments to be reported in detail elsewhere show that Na⁺ injection or tetanic spike discharge in SC cells can result in hyperpolarisations similar to PGH. Thus, we conclude that PGH as well as these other potentials results primarily from the current generated by increases in the rate of electrogenic Na⁺ transport triggered by Na⁺ loading induced by certain chemical and electrical stimuli. The increase in PGH which is seen with hyperpolarisation (Fig. 1c) presumably results from increased Na⁺ loading that would be expected as a result of the increased Na⁺ driving force produced by the hyperpolarisation. Our results also suggest that there is an electrogenic component to the resting potential in SC cells amounting to about −5 mV.

The significance of the potentials reported here is not well understood. Such long-lasting neural signals can have immediate effects on information processing[13,14], and may encode important and unique aspects of previous nervous activity. It is tempting to speculate that these or similar processes may intervene between rapid electrochemical events and more lasting biochemical phenomena. An obvious opportunity for this sort of linkage exists in DRG cells in which both PTH and the initiation of axoplasmic transport[15] may have an important dependency on Ca²⁺. The PGH in SC cells, on the other hand, is clearly associated with an increase in Na⁺ transport and thus ATPase activity. The attendant effects on energy metabolism might be expected to have a variety of biochemical sequellae.

BRUCE R. RANSOM
JEFFERY L. BARKER
PHILLIP G. NELSON

Behavioral Biology Branch,
National Institute of Child Health and Human Development,
National Institutes of Health,
Bethesda, Maryland 20014

1 Nakajima, S., and Takahashi, K., *J. Physiol., Lond.,* 187, 105–127 (1966).
2 Thomas, R. C., *Physiol. Rev.,* 52, 563–594 (1972).
3 Meech, R. W., *Comp. Biochem. Physiol.,* 48, 387–401 (1974).
4 Jansen, J., and Nicholls, J. G., *J. Physiol., Lond.,* 229, 635–655 (1973).
5 Kuno, M., Miyahara, J. T., and Weakly, J. N., *J. Physiol., Lond.,* 210, 839–855 (1970).
6 Ransom, B. R., and Goldring, S., *J. Neurophysiol.,* 36, 879–892 (1973).
7 Koike, H., Mano, N., Okada, Y., and Oshima, T., *Expl Brain Res.,* 14, 489–503 (1972).
8 Peacock, J. H., Nelson, P. G., and Goldstone, M. W., *Devl Biol.,* 30, 137–152 (1973).
9 Ransom, B. R., and Nelson, P. G., in *Handbook of Psychopharmacology,* 2, (edit. by Iversen, L. L., Iversen, S. D., and Snyder, S. H.), 101–127 (Plenum, New York, in the press).
10 Krnjevic, K., *Physiol. Rev.,* 54, 418–540 (1974).
11 Hosli, L., Hosli, E., and Andres, P. G., *Brain Res.,* 62, 597–602 (1973).
12 Thomas, R. C., *J. Physiol., Lond.,* 201, 495–514 (1969).
13 Baylor, D. A., and Nicholls, J., *J. Physiol., Lond.,* 203, 571–589 (1969).
14 Van Essen, D., *J. Physiol., Lond.,* 230, 509–534 (1973).
15 Hammerschlag, R., and Dravid, A., *Trans. Am. Soc. Neurochem.,* 108, (1975).

Activation of a voltage-insensitive conductance by inward calcium current

ELECTRICAL excitability is more complex in cell bodies than in axons. In various nerve cells there is a voltage-sensitive permeability to calcium as well as to sodium and potassium. Furthermore, increased calcium in the cytoplasm of nerve cell bodies increases their potassium permeability. In voltage-clamped *Helix* neurones, Meech and Standen[1,2] reported that a substantial fraction of the potassium conductance activated during a depolarisation depends on an inward calcium current.

We have studied the sensory epithelium of the electroreceptor (ampulla of Lorenzini) of the skate. In this epithelium, which

can generate all-or-none action potentials[3,4] of 60 mV, we have found that the repolarising phase of the action potential results from a late outward current initiated when calcium ions enter the cytoplasm of the excitable cells. Because there is no detectable voltage-sensitive outward current, we conclude that a distinct calcium-activated conductance mechanism is involved.

Skate electroreceptor epithelium consists of a single layer of interspersed receptor and supporting cells (Fig. 1b). The epithelium forms a lobulated sac, or ampulla of Lorenzini, which is connected to an external orifice by a long canal (Fig.
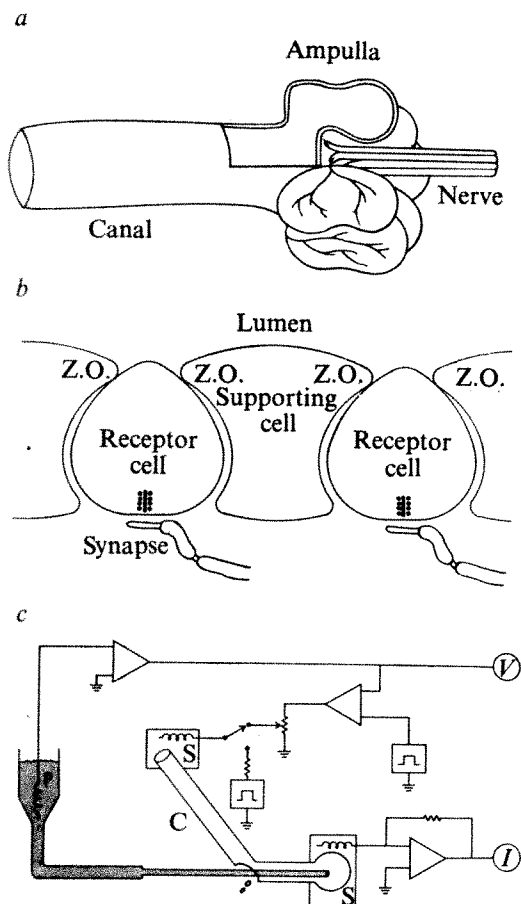
Fig. 1 *a*, Skate ampullary electroreceptor and canal (after Waltman[5]). *b*, Details of two receptor cells in the ampullary epithelium. Occluding junctions (zonulae occludentes, Z.O.) occur between receptor cells and adjacent supporting cells. The basal membranes form characteristic ribbon synapses with the afferent nerve fibres[5]. *c*, Experimental arrangement for current clamping, voltage clamping, and internally perfusing an ampulla. The ampulla with its canal (average diameter 1 mm, average length 7 cm) was removed from a skate (*Raja oscellata* or *R. erinacea*). The open end of the canal was placed in one saline pool (S) and the ampulla in another. Little current flows along the external surface of the canal (C) because it was rinsed with a solution of 526 mM sucrose and 350 mM urea before it was placed in the recording chamber. A 100-μm glass cannula was introduced through a nick in the canal wall. The cannulated portion of the canal was in a Vaseline gap. The uncannulated portion was in air. The saline pools contained cerebrospinal fluid from the cranial cavity or occasionally Fühner's[9] elasmobranch saline. The perfusate (P) was under slight pressure. Effluent was aspirated. All perfusates were made from a control solution containing 428 mM NaCl, 13 mM KCl, 50 mM MgCl$_2$, 10 mM CaCl$_2$, 75 mM urea and 5 mM HEPES at pH 8.1. Ionic exchange inside the ampulla was limited by diffusion through the gelatinous material that fills the individual lobules. When the ampulla was perfused with phenol red, noticeable diffusion into all the lobules required about 15 min. Epithelial current was measured by a current–voltage transducer with output *I*. Epithelial voltage was measured at the perfusion cannula, and controlled using an operational amplifier and a voltage source. Constant current pulses were applied by connecting the electrode at the canal orifice to a second voltage source through a 10^8 Ω resistor.

1a). The canal is a core conductor, the wall of which is electrically linear and has a high resistivity[5]. All cell surfaces abutting on the lumen of the ampulla and its canal are joined by occluding junctions which presumably prevent extracellular leakage of current or ions across the epithelium. The occluding junctions separate the excitable lumenal faces of the receptor cells from their basal faces, which are innervated by afferent fibres of the VIIIth cranial nerve (Fig. 1b).

The electroreceptor epithelium was voltage clamped as shown in Fig. 1c. During lumen-positive voltage steps (which hyperpolarise the lumenal membranes), epithelial current was constant after the capacitative transient. When the lumen was made negative (depolarising the lumenal membranes), however, active currents were observed (Fig. 2a). Excitatory (lumen-negative) voltage steps of moderate amplitude evoked an early current which flowed inward across the lumenal membranes followed by a maintained outward current. At voltages less than 30 mV, multiple peaks of inward current sometimes occurred. We attribute active currents to the receptor cells because of the associated postsynaptic potentials that could be recorded in the afferent nerve fibres. The conductance changes responsible for the active currents are attributed to the lumenal faces of the receptor cells because these currents are dramatically altered by ionic substitutions in the lumen (see below), but are only slightly affected by drastic alterations outside the basal membranes, for example, chelation of calcium with EGTA, or 90% replacement of NaCl with potassium acetate.

When increasingly large excitatory voltage steps were applied across the epithelium, onset of the late outward current became slowed and delayed (Fig. 2a). At about 130 mV lumen-negative, activation of the late outward current was totally suppressed. The remaining early current did not inactivate significantly during the clamping pulse. A current–voltage relationship of the electroreceptor epithelium is shown in Fig. 2b. Excitatory (lumen-negative) voltages are to the right of the vertical axis. For excitatory voltage steps, the peak inward and late outward

Fig. 2 *a*, Currents recorded from skate electroreceptor epithelium during perfusion with the control solution. Current flowing inward across the lumenal membranes of the receptor cells and outward across the basal membranes is defined as inward current and shown downward. Voltage displacements that depolarise the lumenal membranes (lumen-negative) are defined as positive. *b*, A current–voltage relationship plotted from the data in *a*. The vertical axis is placed at the holding potential, which is the potential developed by the epithelium when no current is allowed to flow in the canal. The reversal potential for the early current, obtained by extrapolation of the leakage current (long dashes), is also the potential at which the late outward current is suppressed. *c*, Current–voltage relationships obtained from the same preparation after the free calcium concentration in the lumen had been reduced by perfusion with EGTA.
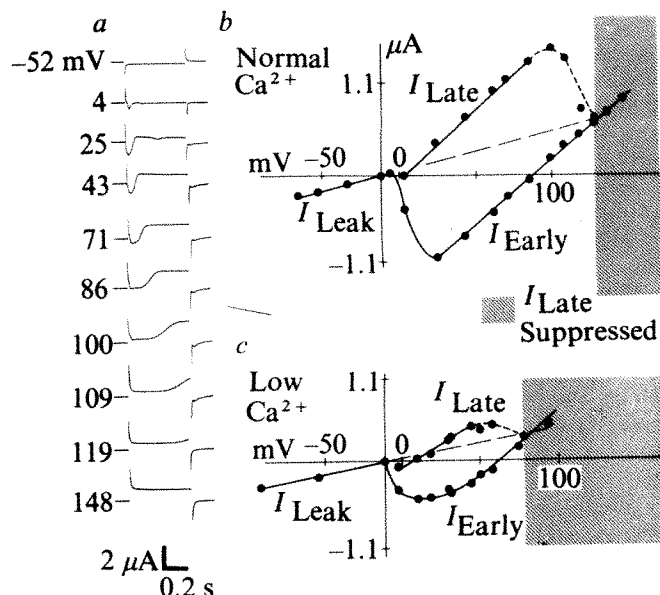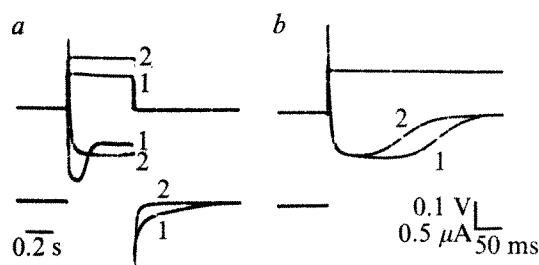
Fig. 3 *a*, Repolarisation tail currents recorded from skate electroreceptor epithelium. Two sweeps with voltage steps of different amplitude are superimposed. The smaller stimulus, 1, evokes a late outward current and a repolarisation tail current lasting 600 ms. The larger stimulus, 2, exceeds the reversal potential for the early current, and evokes no late current and no prolonged repolarisation tail. *b*, Facilitated onset of the late outward current by a conditioning stimulus. Two uniform voltage steps are applied 2 s apart and the currents are superimposed. The early current is identical for the two stimuli, but the late outward current evoked by the conditioned stimulus, 2, occurs at much shorter latency than that evoked by the conditioning stimulus, 1.

currents were linear functions of voltage over a broad range. For lumen-positive steps, current was also linearly related to voltage, the slope of the line being the leakage resistance.

We were able to demonstrate that the resistance immediately after the onset of an excitatory voltage displacement (instantaneous resistance) was equal to the leakage resistance. (This demonstration was possible because the form of the capacitative transient did not depend on the absolute voltage or the epithelial conductance. Capacitative transients could therefore be subtracted from the total current.) Moreover, experiments with superimposed pulses showed that the instantaneous resistance of the epithelium during maintained early and late currents was equal to the corresponding slope resistance. Knowing these instantaneous resistances, it is possible to determine the reversal potential for the early current. This reversal potential, about 130 mV, was equal to the suppression potential for the late outward current.

Early current in skate electroreceptors is carried by calcium as shown by the following observations: (1) The action potential was unaffected when the lumen of the ampulla was perfused with 0.1 mM TTX. (2) The action potential was abolished by perfusion with cobalt. (100 mM $CoCl_2$ was prepared by isosmotic substitution for NaCl in the control saline. Because of diffusion delays, the effective concentration at the ampullary epithelium must have been substantially lower.) (3) Inward current was abolished by a zero calcium solution containing EGTA. (20 mM EGTA and 60 mM HEPES were brought to *p*H 8.1 with NaOH and isosmotically substituted for NaCl.)

The current–voltage relationship of Fig. 2*c* was obtained during perfusion with a solution containing normal (10 mM) Ca, 20 mM EGTA and 60 mM HEPES. The data were taken after about 15 min, before ionic equilibration was complete. Although the free calcium concentration is not known, the reversal potential for the early current was clearly reduced, as was the magnitude of the early current at all voltages. Significantly, the suppression potential for the late outward current still corresponded to the reversal potential for the early current. These observations suggest that the early current is carried by calcium, and that the late current is not activated unless there is a net influx of calcium into the cytoplasm. This interpretation is corroborated by the fact that no delayed rectification was seen when the epithelial action potential was abolished by perfusion of the lumenal membranes with EGTA or cobalt.

When the epithelium is repolarised following activation of the late outward current, a tail current lasting about 600 ms is observed (Fig. 3*a*, trace 1). Prolonged tail currents are not observed when the excitatory voltage stimulus exceeds the reversal potential for the early current and there is no late current (trace 2). Prolonged tail currents are augmented, but

cannot be shortened by repolarising the epithelium to increasingly lumen-positive voltages. These observations suggest that the prolonged tail current flows in a voltage-insensitive conductance that is activated by an early inward current.

The absence of any voltage-sensitive component of the slow current requires that a different type of conductance mechanism, presumably a distinct macromolecule, be responsible for terminating the action potential in skate electroreceptors. An important property of this calcium-activated conductance is that it undergoes facilitation. In Fig. 3*b*, two identical pulses were applied 2 s apart. The conductance of the epithelium returned to normal between pulses and the size of the inward current evoked by the two pulses was uniform. The onset of the late outward current, however, occurred considerably earlier during the second stimulus. This effect of a conditioning stimulus lasted about 10 s. It was not observed when the conditioning stimulus exceeded the reversal potential for the early current. Residual calcium may therefore have been involved. Facilitated onset of a calcium-activated hyperpolarising conductance may mediate sensory accommodation in skate electroreceptors[7].

Evidence for a calcium-activated hyperpolarising conductance has been adduced for each of several nerve cell bodies that have been studied in this respect[6,8]. It seems likely that the underlying molecular basis is the same in these cells as in skate electroreceptors, although in neurones experiments are complicated by the presence of a voltage-sensitive late conductance. Calcium-activated conductances could have a major role in the regulation of electrical activity in cell bodies, particularly if they exhibit the facilitated onset we have described in skate electroreceptors.

W. Clusin
D. C. Spray
M. V. L. Bennett

Albert Einstein College of Medicine
Bronx, New York 10461 and
Marine Biological Laboratory
Woods Hole, Massachusetts 02543

1 Meech, R. W., and Standen, N. B., *J. Physiol., Lond.*, **237**, 43–44 (1974).
2 Meech, R. W., and Standen, N. B., *J. Physiol., Lond.* (in the press).
3 Waltman, B., *Acta physiol. scand.*, **74**, 29A (1968).
4 Obara, S., and Bennett, M. V. L., *J. gen. Physiol.*, **60**, 534–557 (1972).
5 Waltman, B., *Acta physiol. scand. suppl.*, **264**, 1–60 (1966).
6 Meech, R. W., *J. Physiol., Lond.*, **237**, 259–277 (1974).
7 Murray, R. W., *J. exp. Biol.*, **39**, 119–128 (1962).
8 Jansen, J. K. S., and Nicholls, J. G., *J. Physiol., Lond.*, **229**, 635–655 (1973).
9 Fühner, H., *Z. für Allgemeine Physiol.*, **8**, 485–491 (1908).

Selective elimination of taste responses to sugars by proteolytic enzymes

INTERACTION between taste stimuli and taste receptors has been suggested as the initial event preceding the taste cell excitation, and, based on a number of biochemical investigations, the receptors are presumed to be proteinacious in nature[1–3]. For example, a protein which combines with sweet substances was extracted from bovine[4] and rat tongues[5]. Direct evidence for the ability of the extracted protein to bind with sugars has come from experiments using ^{14}C-labelled fructose and polyacrylamide gel electrophoresis or Sephadex gel filtration[6,7]. The presence of a sugar-binding receptor in taste buds has also been indicated by an experiment in which suspensions of papillae containing taste buds were found to bind more ^{14}C-sucrose than those without taste buds. Furthermore this binding ability could be abolished by heating[8]. Therefore, if taste receptors for sweet substances are composed of a protein, the neural responses to sugars would be eliminated after digestion of the receptor protein by proteolytic enzymes. To check the above hypothesis, various proteases were applied to the rat tongue surface and impulses from the taste nerve were recorded. In addition,
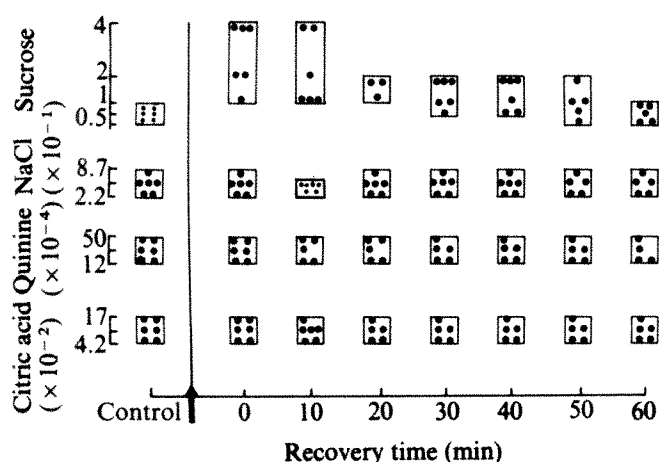
Fig. 1 Integrated responses of rat chorda tympani to stimulation of the tongue with 1 M sucrose, 0.02 M quinine–HCl, 0.01 M HCl, and 0.05 M NaCl before (control) and after treatment with 2 % pronase E for 30 min. The two right-hand columns indicate the responses after rinsing the tongue with water. Female Sprague–Dawley rats (150–250 g) were anaesthetised by intra-peritoneal injection of sodium amobarbital (70 mg per kg body weight). Integrated responses to taste stimuli were recorded from the chorda tympani[9]. In each experiment, the integrated res-ponses to four basic taste stimuli (0.05 M NaCl, 0.01 N HCl, 0.02 M quinine hydrochloride and 1.0 M sucrose) were recorded first as controls. Subsequently, one of the enzymes (with pH adjusted with phosophate buffer solutions at 30–50 °C) was applied to the rat tongue through a glass flow-chamber for 20 min or more, after which the tongue was rinsed with 300–400 ml tapwater and the above series of stimulations repeated. Com-mercially obtained enzymes were used without further purifica-tion.

Fig. 3 Changes in taste thresholds in human subjects by appli-cation of Pronase E (2 %, 10 min, at arrow). Each box indicates the range of the taste threshold concentration (%) for sucrose, NaCl, quinine–HCl and citric acid before and after application of Pronase. Note the recovery to normal of the elevated threshold for sucrose. For sensory examinations a filter paper immersed in Pronase solution at the same con-centration as that applied to the rat tongue was placed on the tongue of human subjects for a period of 10 min. After the filter paper had been taken off, the tongue was rinsed with distilled water, and taste solution applied with a pipette to the same area. The tongue was rinsed with distilled water after each stimulation with a taste solution.

an alteration of the taste threshold produced by the enzymes were also examined in human subjects.

Application of various proteolytic enzymes such as trypsin (pH 7.0), papain (pH 7.0) and neutral (pH 7.0) and acidic (pH 4.7) proteases to the tongue surface for more than an hour did not produce any effect on the neural responses to the four basic taste stimuli (Fig. 2). On the other hand, when Pronase E (pH 7.0) or semi-alkaline pro-tease (pH 8.0) was applied to the tongue for 20 min, the chorda tympani response to 1 M sucrose was suppressed (Figs 1 and 2), whereas responses to the other three taste stimuli were hardly affected (Fig. 1). Similar suppression of responses to various sweet substances was also observed. Responses to 1 M glucose, 1 M fructose, 0.8 M sorbitol

and even 0.005 M saccharin were completely eliminated by the previous application of Pronase E or semi-alkaline protease, and responses to 0.8 M glycine and 0.8 M DL-alanine, which produce sweet taste in man, were also depressed markedly. By rinsing the tongue with water for a period of 30 min or more after the enzyme application, the eliminated responses to the sweet substances were com-pletely recovered to the same magnitude as found for controls (Figs 1 and 2). In contrast, Pronase E, which was
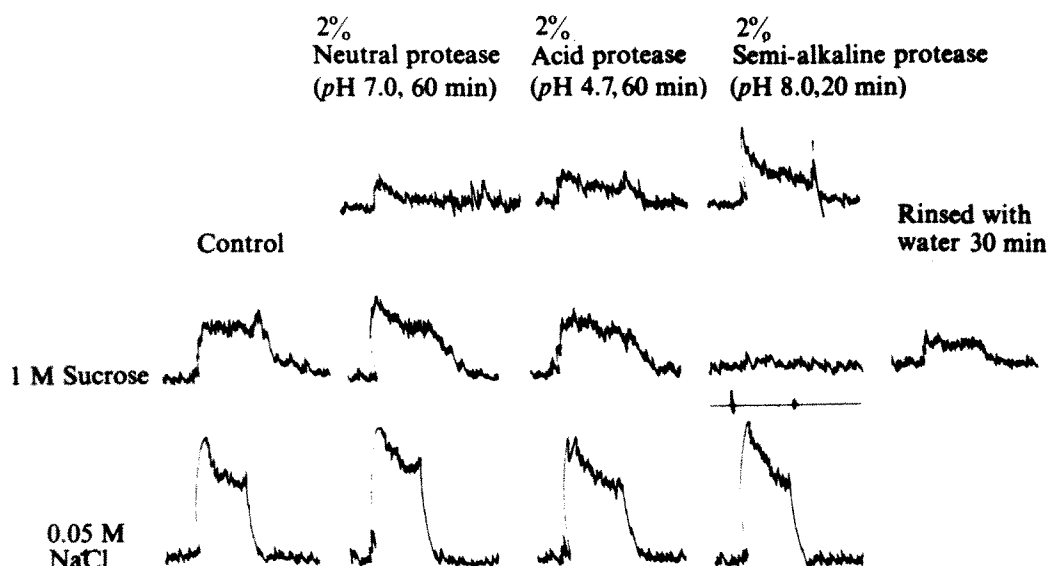
Fig. 2 Integrated res-ponses of the rat chorda tympani to 1 M sucrose and 0.05 M NaCl (left column). The three middle columns indicate the responses after treat-ment with 2 % neutral, acid and semi-alkaline proteases and the right column shows those after rinsing the tongue with water. On–off markers in sucrose response indicate the period of application of taste solution to the tongue.

inactivated by heating at 80 °C for 5 or 10 min, produced little effect on the integrated response to sucrose.

Essentially the same effect for Pronase as that obtained by the neural experiments was demonstrated in the sweet sensation of man (Fig. 3). The mean ($\pm$ s.d.) threshold concentration for sucrose in man treated with Pronase was $2.8 \pm 1.3\%$ (w/v), which is about five times greater than normal ($0.58 \pm 0.32\%$), and the difference is significant ($0.01 > P > 0.001$; Student's t test). The elevated threshold reverted to normal by rinsing the tongue with water for 20–60 min. On the other hand, thresholds for the other three tastes remained within the normal range ($0.44 \pm 0.21\%$ for NaCl, $0.003 \pm 0.0013\%$ for quinine hydrochloride, and $0.099 \pm 0.053\%$ for citric acid) before and after application of Pronase (Fig. 3). The present sensory data confirmed the observations on the neural response in the rat.

In the present study it has been demonstrated that the proteolytic enzyme selectively suppresses rat chorda tympani responses to sweeteners without affecting those to other kinds of taste stimuli. An elevated threshold for sugar by application of the enzyme was also found in the sensory examinations. Thus, the neurophysiological experiments on rats qualitatively duplicate the sensory data in men. Both kinds of study may indicate that the sugar-binding ability of the receptor is depressed, possibly as a result of digestion of the receptor protein by the enzyme. Consequently, our findings support the conclusion, derived from biochemical investigations[4-8], that the sugar receptor molecules are composed of a protein. The recovery of taste responses after rinsing the proteolytic enzyme with water may be the result either of the strong repairing action of the protein or of a rapid turnover mechanism of the receptor protein synthesised in taste cells.

Giroux and Henkin[10] have reported that proteolytic enzymes produce a non-selective increase in the threshold for each of the four basic tastes in man, and that alterations in taste acuities lasted for as long as 12 h after application of Pronase. Their results hardly agree with those from our experiments. This difference may be the result of a difference in the dosages used. In their examinations the total dosage of Pronase was 6 mg in a buffer solution, and the enzyme was thoroughly circulated within the oral cavity, which produced sensations of tingling and burning. In our sensory examinations the total dosage of Pronase absorbed in a filter paper placed on the tongue was about 1 mg. The actual concentration affecting taste buds may be less, because of dilution by saliva. This small dosage produced a light astringent taste and depressed the sugar response alone. Tingling and burning sensations after the enzyme treatment in the former experiments may suggest that not only the protein composing the sugar receptor but also the taste cell membrane responsible for other stimuli would be simultaneously digested by a strong proteolysis, leading to long lasting alteration in the thresholds not only for the sweet taste but also the other three tastes.

Y. Hiji

Department of Physiology,
Kumamoto University Medical School,
Kumamoto, Japan

1 Beidler, L. M., *J. gen. Physiol.*, **38**, 133 (1954).
2 Schallenberger, R. S., and Acree, T. E., *Nature*, **216**, 480 (1967).
3 Kubota, T., and Kubo, I., *Nature*, **233**, 97 (1969).
4 Dastoli, F. R., and Price, S., *Science*, **154**, 904 (1966).
5 Hiji, Y., Kobayashi, N., and Sato, M., *Comp. Biochem. Physiol.*, **39B**, 367 (1971).
6 Hiji, Y., and Sato, M., in *Olfaction and Taste*, **4** (edit. by Schneider, D.), 221 (Wissenschaftliche, Verlagsgesellschaft, Stuttgart, 1972).
7 Hiji, Y., and Sato, M., *Nature new Biol.*, **244**, 91 (1973).
8 Cagan, H. R., *Biochim. biophys. Acta*, **252**, 199 (1971).
9 Yamashita, S., and Sato, M., *J. cell. Physiol.*, **66**, 1 (1965).
10 Giroux, E. L., and Henkin, R. I., *Life Sci.*, **10**, 361 (1971).

Evidence for phosphofructokinase in chloroplasts

ATTEMPTS to demonstrate phosphofructokinase activity in chloroplasts have so far been unsuccessful[1-4], thus raising the question of whether the transitory starch formed in the chloroplast during photosynthesis can subsequently be metabolised to triose phosphates within the chloroplast. Such a conversion would seem beneficial if the products of starch degradation are required to move freely from the chloroplast to the cytoplasm, since triose phosphates, but not hexose phosphates, are actively and rapidly transported across the chloroplast membrane[5]. The apparent absence of phosphofructokinase from chloroplasts was also discouraging since this enzyme is considered one of the principal regulatory enzymes of carbohydrate metabolism[6] and the regulatory properties of the plant enzyme have been shown consistent with the control of starch metabolism[7]. Investigations in this laboratory have now demonstrated that chloroplasts do contain phosphofructokinase activity.

Spinach chloroplasts were isolated by homogenising 24 g leaves for 5 s in 120 ml of the isolation medium described by Lilley *et al.* (ref. 8). The homogenate was filtered through Miracloth, centrifuged at 2,500g for 80 s and the pellets of chloroplasts broken in 30 ml of a solution of pH 7.7 consisting of 5 mM $MgCl_2$, 5 mM dithioerythritol and 1 mM EDTA and containing 1 g Polyclar AT. A sample of the supernatant was collected as representative of the cytoplasmic fraction; dithioerythritol was added to 5 mM, the pH adjusted to 7.7 and 1 g Polyclar AT stirred in. After 10 min these chloroplast and cytoplasmic samples were centrifuged at 22,000g for 20 min, the supernatants collected and that from the chloroplasts was concentrated through a Diaflo XM-50 filter to 4 ml. Samples (2 ml) of each of the concentrated chloroplast extract and cytoplasmic fraction were dialysed for 3 h against 1 l of a solution of pH 7.7 containing 5 mM imidazole-HCl, 1 mM $MgCl_2$, 1 mM dithioerythritol and 0.5 mM EDTA.

The activity of phosphofructokinase is compared in Table 1 with the activities of three marker enzymes: non-reversible glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is restricted to the cytoplasm[9], the NADP-linked reversible GAPDH found only in the chloroplast[2], and fructose-1, 6-diphosphate aldolase (FDP-aldolase) which occurs both in chloroplasts and the cytoplasm[2]. The specific activity of non-reversible GAPDH in the chloroplast extract was only 2% of that in the cytoplasmic fraction, indicating that cytoplasmic contamination of these chloroplasts isolated in aqueous media was minimal. In contrast, considerable phosphofructokinase activity was detected in the chloroplast extract, the specific activity being 25% of that in the cytoplasmic fraction. The clear difference between the ratios of the specific activities of these two enzymes (Table 1) provides strong evidence that the phosphofructokinase activity detected in the chloroplast extract did not originate from cytoplasmic contamination of chloroplasts during isolation but rather represents an authentic localisation of phosphofructokinase with the chloroplast.

The activities of chloroplast GAPDH and FDP-aldolase (Table 1) show that a considerable number of the chloroplasts were broken during isolation; assuming all the chloroplast GAPDH activity and 65% of the FDP-aldolase activity are located in chloroplasts[2] the results in Table 1 show that enzyme loss from chloroplasts was between 65 and 80%. Consequently the total chloroplast phosphofructokinase activity shown in Table 1 is probably only a fraction of the true value.

Several lines of evidence confirm the presence of phosphofructokinase activity. The enzyme activity was dependent on both fructose-6-phosphate and ATP; no NADH oxidation was observed in the absence of either substrate if the preparation was dialysed before assay. Both phosphoenolpyruvate and

Table 1 Activities of phosphofructokinase and three marker enzymes in a chloroplast extract and cytoplasmic fraction from spinach leaves

| | Chloroplast extract | | Cytoplasmic fraction | | Ratio: |
	Total activity*	Specific activity†	Total activity*	Specific activity†	s.a. chloroplast / s.a. cytoplasm
Phosphofructokinase	1.9	0.25	31	1.0	0.25
Non-reversible GAPDH	0.40	0.05	72	2.4	0.02
Chloroplast GAPDH	320	45	1200	39	1.1
FDP-aldolase	110	14	310	10	1.4

* Measured as μmol per h per 10 g fresh weight of leaves.
† Expressed as μmol per mg protein per h.

Enzyme activities were determined at 22 °C. The reaction mixtures for phosphofructokinase contained, in a final volume of 1 ml, 50 μmol imidazole-HCl buffer (*p*H 7.7), 2.5 μmol $MgCl_2$, 2.5 μmol dithioerythritol, 1.5 μmol D-fructose-6-phosphate, 0.5 μmol ATP, 0.08 μmol NADH, 1 unit aldolase, 12 units triosephosphate isomerase, 1 unit α-glycerophosphate dehydrogenase and 50–100 μl extract. The composition of reaction mixtures for the determination of FDP-aldolase and chloroplast GAPDH activities were based on those described earlier[2]; the buffers used in the present experiment were imidazole-HCl (*p*H 7.7) and triethanolamine-NaOH (*p*H 8.0) respectively. Non-reversible GAPDH was assayed as described previously for cell-free plant extracts[9]. All assay enzymes were dialysed before use to remove $(NH_4)_2SO_4$. Activities were calculated from changes in extinction of reaction mixtures at 340 nm measured spectrophotometrically. Protein was determined by the Biuret procedure as described previously[9].

relatively high concentrations of ATP inhibited the chloroplast phosphofructokinase activity: phosphoenolpyruvate at 0.1 mM produced more than 70% inhibition, while the activity with 4.8 mM ATP was less than half that with the optimum ATP concentration of 0.1 mM. Inhibition with increased levels of ATP is a common property of phosphofructokinase[6] and sensitivity to inhibition by phosphoenolpyruvate is a notable feature of plant phosphofructokinase[7]. Some of these regulatory phenomena may explain why phosphofructokinase activity was not found in chloroplasts in earlier studies[1-4] particularly if ATP levels used in those assays were too high.

Preliminary experiments in which intact chloroplasts were added to a solution containing the substrates for phosphofructokinase showed that the product fructose-1,6-diphosphate was not formed when the solution was isotonic (containing 0.33 M sorbitol) and the chloroplasts remained intact, but product was formed at a rate of approximately 2 μmol per mg chlorophyll per h when sorbitol was omitted and the chloroplasts were broken. These results not only confirmed that the phosphofructokinase was contained within the chloroplast, but also demonstrated an activity compatible with the amount of starch expected to be metabolised in chloroplasts. It is not inconceivable that chloroplast phosphofructokinase catalyses a regulatory step in the degradation of starch in the chloroplast. Efforts are now being made to separate and partially purify the chloroplast and cytoplasmic phosphofructokinases from leaves with a view to relating their regulatory properties to the carbon metabolism of photosynthesis. Initial results are encouraging.

G. J. KELLY
E. LATZKO

Abteilung Chemische Pflanzenphysiologie,
Technische Universität München,
8050 Weihenstephan, Germany

1 Smillie, R. M., *Can. J. Bot.*, **41**, 123–154 (1963).
2 Latzko, E., and Gibbs, M., *Z. PflPhysiol*, **59**, 184–194 (1968).
3 Schnarrenberger, C., and Oeser, A., *Eur. J. Biochem.*, **45**, 77–82 (1974).
4 Kachru, R. B., and Anderson, L. E., *Pl. Physiol., Lancaster*, **55**, 199–202 (1975).
5 Heldt, H. W., and Rapley, L., *FEBS Lett.*, **10**, 143–148 (1970).
6 Lowry, O. H., and Passonneau, J. V., *Archs exp. Path. Pharmak.*, **248**, 185–194 (1964).
7 Kelly, G. J., and Turner, J. F., *Biochim. biophys. Acta*, **208**, 360–367 (1970).
8 Lilley, R. McC., Walker, D. A., and Holborow K., *Biochim. biophys. Acta*, **368**, 269–278 (1974).
9 Kelly, G. J., and Gibbs, M., *Pl. Physiol., Lancaster*, **52**, 111–118 (1973).

Localisation of plasma α₂HS glycoprotein in mineralising human bone

THE protein of the organic matrix of bone consists mainly of collagen in association with a small proportion of non-collagenous proteins. When bone is decalcified, much of the non-

collagenous protein can be solubilised in conditions of neutral *p*H but the collagen remains insoluble. Extracts of decalcified bone prepared in this manner contain small amounts of plasma proteins which derive in part from the presence of blood vessels within the tissue[1,2]. There is evidence, however, that some plasma proteins, including albumin, form an integral part of the calcified matrix[3]. When ^{14}C-glucosamine or ^{14}C-glucosamine-labelled rabbit total plasma protein is injected into a rabbit, some activity is incorporated into the bone[4]. Using the procedure of Herring[5] to separate the EDTA-soluble proteins of the bone matrix, most of the activity was shown to be present in the less acidic glycoprotein fraction, much of this in one glycoprotein which was also present as a minor component of blood[4]. This G2B glycoprotein had the electrophoretic mobility of an α globulin and a molecular weight by SDS gel electrophoresis[6] of about 50,000. Studies by Ashton *et al.* (private communication) support the view that the G2B glycoprotein they have investigated in rabbit[4] and bovine bone[6] is the analogue of human α₂HS glycoprotein.

In this report we provide evidence by immunoprecipitation and passive haemagglutination techniques that α₂HS glycoprotein (an α globulin of estimated molecular weight 49,000 by ultracentrifugal analysis[10] present in human plasma at a concentration of about 60 mg dl^{-1}) is concentrated extravascularly in the matrix of normal human bone and can be located in areas of mineralisation in bone using an immunofluorescent antibody-staining technique.

The non-collagenous proteins of normal adult human cortical bone were isolated by sequential extraction of the powdered bone with 0.5 M EDTA at *p*H 7.6. The concentrated extract, dialysed free of mineral salts, and human plasma each gave a single precipitin line of complete identity when they were diffused against the antiserum to plasma α₂HS glycoprotein. The antiserum used in these studies was raised in rabbits against human plasma α₂HS glycoprotein and was specific for the latter, as judged by immunoelectrophoresis and double diffusion against plasma proteins. Tanned sheep erythrocytes, coated with the non-collagenous bone protein as described previously[1], agglutinated in the presence of antiserum to α₂HS glycoprotein but not with antisera to two other human plasma α globulins, α₁ antitrypsin and α₂ macroglobulin (Table 1). The latter two proteins are present in plasma at concentrations approximately three times higher than α₂HS glycoprotein in normal individuals. This suggested that the identification of only the latter protein in bone in these conditions did not derive from the presence of blood within the tissue. The antisera to the three α globulins were each adsorbed with lyophilised bone protein and then incubated with human plasma protein-coated tanned sheep erythrocytes. These preparations had negligible effect (Table 1) on the haemagglutination titres of two of the antisera but the activity of that against α₂HS glycoprotein was destroyed. By radial immunodiffusion in an agarose gel containing antibody, the concentration of α₂HS
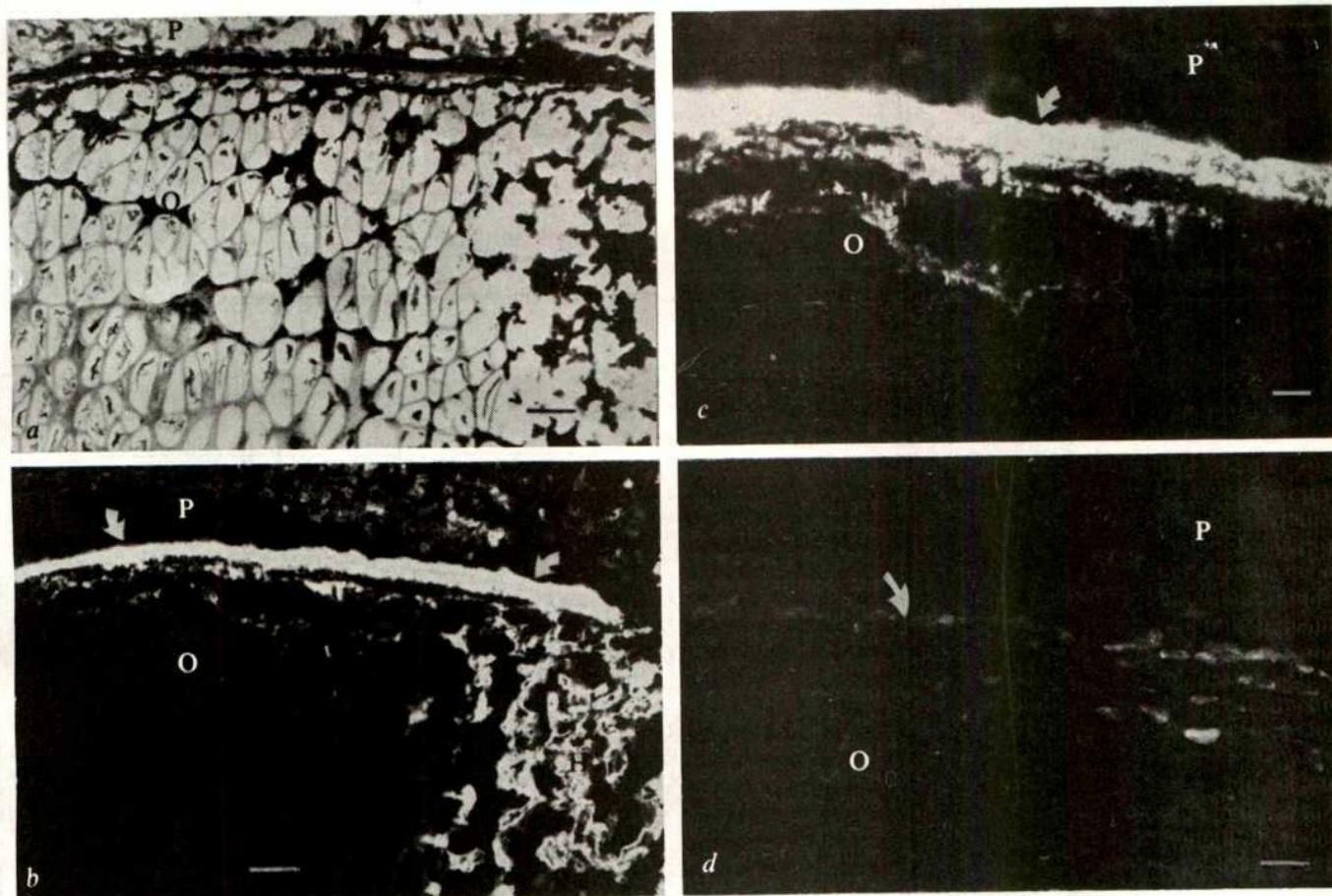
Fig. 1 Longitudinal 6 μm thick sections of femur. Frozen sections were fixed for 5 min in formaldehyde as described previously[8]. Sections were then washed in phosphate-buffered saline (PBS) for 30 min. *a*, Stained with von Kossa's reagent and counterstained with haematoxylin and chromotrope. A band of brown/black staining (arrows) in the periosteum (P) overlays staining for calcium in the hypertrophic cartilage which is either heavily ossified (H) or partially ossified (O). *b–d*, Immunohistochemical localisation of α_2HS glycoprotein. Sites of binding of rabbit antibody Fab' to α_2HS glycoprotein in tissue sections were detected by subsequently using fluorescein isothiocyanate-labelled pig antibody Fab' directed against rabbit Fab'. Thus sections were initially treated with pepsin digests (at 1.6 mg ml⁻¹) of 33% (NH₄)₂SO₄ immunoglobulin G-rich fractions isolated from the antiserum to α_2HS glycoprotein (*b* and *c*) or non-immune rabbit serum (control); *d*, as described previously[9]. Sections were washed in PBS and finally stained with Fab' isolated from a pig antiserum to rabbit Fab', and labelled with fluorescein isothiocyanate at a concentration of 2 mg ml⁻¹. Sections were finally washed, mounted and examined (for details see ref. 9). *b* and *c*, Intense green extracellular fluorescence can be seen in the calcified band in the periosteum (arrows) and in the heavily ossified sites in the shaft (H) is magnified region of *b*. Less intense staining is present in sites of partial ossification (O). *d*, No intense green fluorescence was detected and only weak non-specific cellular staining was seen. Comparable sites are indicated as in *b* and *c*. Scales: *a* and *b*, 50 μm; *c* and *d*, 20 μm.

glycoprotein was estimated as 0.1% of the organic matrix of bone.

To establish whether the protein was incorporated into bone during the process of mineralisation, attempts were made to localise the protein by immunohistochemical techniques. The tissue used was the femur of a human foetus aged approximately 12 weeks (obtained after a therapeutic abortion). The femur was placed in a solution of 7% gelatin and 0.09% NaCl at 30 °C and then rapidly frozen in liquid nitrogen before final storage at −20 °C. Frozen sections were fixed and then incubated with a pepsin digest of either rabbit anti-(human α_2HS glycoprotein) immunoglobulin G or a rabbit non-immune globulin as control, and then with the fluorescein-labelled Fab' fragment of a pig antibody to rabbit immunoglobulin G Fab'.

Some sections were fixed and stained by von Kossa's method for calcium, being counterstained with haematoxylin and chromotrope. Staining for calcium revealed a broad band of calcification in the periosteum adjacent to the hypertrophic cartilage of the shaft (Fig. 1*a*). There was also extensive calcification within the shaft and early signs of calcification in the hypertrophic cartilage. Wherever extracellular calcium was detected we observed very intense green fluorescent staining in sections which had been initially treated with the digest of the immune globulin (Fig. 1*b* and *c*). The intensity of this immunohistochemical staining was greatest where staining with von Kossa's reagent was most concentrated. Sections which had been treated with the control non-immune immunoglobulin digest showed almost no staining associated with extracellular

Table 1 Haemagglutination titres of various antisera against three plasma α globulins

| Protein | Plasma concentration | Reciprocal heamagglutination titre | | |
| | | Bone protein-coated cells | Serum protein-coated cells | |
			Control	Adsorbed
α_2 macroglobulin	180 mg dl⁻¹	<4	2048	1024
α_1 antitrypsin	150 mg dl⁻¹	<4	512	512
α_2 HS glycoprotein	60 mg dl⁻¹	512	128	<4

*Average concentrations in adult human plasma of three α globulins. Antisera against those proteins were incubated with tanned sheep erythrocytes coated with human bone proteins; only antiserum against α_2HS glycoprotein caused haemagglutination. If these antisera were first absorbed with protein this inhibited the capacity of the antiserum against α_2HS glycoprotein to haemagglutinate tanned sheep erythrocytes coated with either bone protein (not shown) or serum protein compared with the non-adsorbed antiserum control.

sites, including those where calcification had taken or was taking place (Fig. 1d). These control specimens did exhibit a very low level of non-specific green fluorescent cellular staining of the kind also seen in the test sections.

These results show therefore that a plasma protein, α_2HS glycoprotein, is a constituent of both foetal and adult human bone matrix and is incorporated and concentrated into bone presumably through interactions with one of the other components of the mineralising matrix. The function of this protein in bone tissue has not yet been established but it has been observed[7] that human plasma α_2HS glycoprotein possesses opsonic properties, as demonstrated by its capacity to promote increased phagocytosis of *Staphylococcus aureus* and *Escherichia coli* by human neutrophils *in vitro*. This would account for our own observations that plasma levels of α_2HS glycoprotein, measured by radial immunodiffusion, fall markedly after various surgical procedures (I. R. D., unpublished); in the case of surgical damage to bone, plasma levels of this glycoprotein, corrected for plasma volume changes, fall to as little as half their pre-operative values. There is no evidence that this protein is present in areas where pre-osseous cartilage is degraded so that, if the opsonic property is utilised, it will most likely be in mediating the eventual resorption of the bone.

We thank Dr E. Kodicek for advice and support, and Dr C. Alper and Mr D. Evans for antisera. This work was supported in part by the NIH.

I. R. DICKSON

Strangeways Research Laboratory,
Wort's Causeway, Cambridge, UK

A. R. POOLE

Division of Immunology,
Department of Pathology,
University of Cambridge, UK

A. VEIS

Northwestern University Medical School,
303 East Chicago Avenue,
Chicago, Illinois 60611

[1] Dickson, I. R., *Calcified Tiss. Res.*, **16**, 321-333 (1974).
[2] Burchard, J., Havez, R., and Dautrevaux, M., *Bull. Soc. chim. Biol.*, **48**, 851-861 (1966).
[3] Ashton, B. A., Herring, G. M., Owen, M. E., and Triffitt, J. T., *Israel J. med. Sci.*, **7**, 409-411 (1971).
[4] Triffitt, J. T., and Owen, M., *Biochem. J.*, **136**, 125-134 (1973).
[5] Herring, G. M., Ashton, B. A., and Chipperfield, A. R., *Prepar. Biochem.*, **4**, 179-200 (1974).
[6] Ashton, B. A., Triffitt, J. T., and Herring, G. M., *Eur. J. Biochem.*, **45**, 525-533 (1974).
[7] van Oss, C. J., Gillman, C. F., Bronson, P. M., and Border, J. R., *Immun. Commun.*, **3**, 329-355 (1974).
[8] Poole, A. R., Dingle, J. T., and Barrett, A. J., *J. Histochem. Cytochem.*, **20**, 261-265 (1972).
[9] Poole, A. R., in *Methological Developments in Biochemistry*, **4** (edit. by Reid, E.), 1 (Longmans, London, 1974).
[10] Schmid, K., and Bürgi, W., *Biochim. biophys. Acta*, **47**, 440-453 (1961).

Propagation of human wart virus in tissue culture

HUMAN wart (papilloma) virus (HWV) is an authentic human tumour virus. Based on morphology, it has been classified as a member of the papova group[1] of animal tumour viruses. Using electron microscopy, HWV has been found to be associated with human skin warts[2], laryngeal and venereal papillomas[3,4] and epidermodysplasia verruciformis[5]. Moreover, experiments done with human volunteers at the beginning of this century[6-8], showed that filtered extracts of warts, laryngeal and venereal papillomas can cause skin warts in man. Since these early experiments, there has been little increase in our knowledge of HWV as a causative agent of either benign tumours or those which can progress to malignancy[5,9], mainly because of the lack of a system in which to propagate HWV *in vitro*[10]. This report describes the propagation of HWV in a tissue culture system.
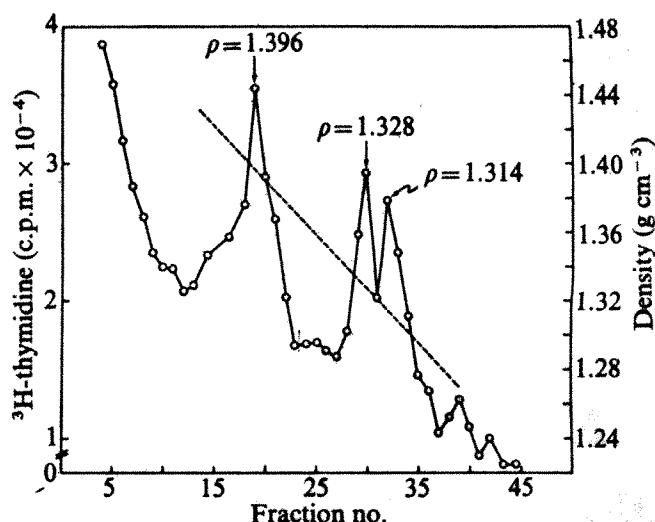
Fig. 1 Isopycnic centrifugation of HWV grown in tissue culture. BE cells were infected with HWV that has been passaged in tissue culture 11 times. ^{3}H-thymidine (2 µCi ml^{-1}) was added to the culture fluid 2 h after infection. Rounded, detached cells along with culture medium were collected 5 d later; cells were disrupted by repeated freeze-thawing, clarified at 800 r.p.m., and virus was concentrated by centrifugation (SW27; 2 h, 25,000 r.p.m.). Resuspended viral pellet was centrifuged in CsCl (ρ 1.32 g cm^{-3}) in an SW50 rotor for 72 h at 40,000 r.p.m.; fractions were collected from the bottom of the tube. Gradient density was determined by measuring refractive index of various fractions.

HWV is epitheliotropic. It infects epidermal cells, a wart probably being a clone of transformed descendants[11] of a single infected cell. Virus progeny are produced in epithelial cells *in vivo*[2]. Our first choice was, therefore, to select a human epithelioid cell strain in which to attempt virus propagation *in vitro*. We have used a cell strain established by Dr M. Bean (Bean epithelial, BE) which, to our knowledge, is unique, as no other epithelioid cell strains have been established from human skin.

Virus was obtained from surgically removed samples of human skin warts, collected in tissue culture medium, incubated at 37 °C for 27 h, and either stored at -80 °C or used immediately. Pooled samples of wart tissue were minced and homogenised with carborundum with a mortar and pestle. Suspensions (10% w/v) were prepared according to a procedure described by Pass and Marcus[12].

To infect BE cells successfully *in vitro*, it was necessary to use cells in the logarithmic phase of growth and to allow the virus to absorb to the cells at low pH (6.5), and then to stress the cells by raising the pH rapidly to an alkaline level (8.0), after which the pH was allowed to fall slowly in a CO_2 incubator to pH 7.0-7.2. Alkaline pH stress was repeated 24 h after infection, and the HWV was thus successfully passaged 14 times in series. Unless cells were treated each time with these three successive pH stresses, serial passage of HWV with cytopathic effect (CPE) failed. The following changes were considered to constitute CPE. At 4-5 d after infection many rounded cells appeared which, in stained preparations, were seen to have intranuclear inclusions accompanied by nuclear vacuolisation. Infected cultures at this time also contained many clear areas surrounded by rounded cells that could be shown by immunofluorescence to contain viral antigens. Rounded cells eventually became detached and died; however, even with continued incubation for 3-4 weeks, only about 50% of the adjacent flattened cells showed similar changes. When the rounded cells were shaken off and examined by indirect immunofluorescence, using hyperimmune rabbit antisera prepared against virus purified from human warts, 90% showed positive nuclear fluorescence. When the remaining attached cells were removed with trypsin and similarly examined, 2-4% were positive. Mock-infected controls treated

in parallel with infected cultures showed neither cytopathic changes nor positive fluorescence.

As progression of the cytopathic changes to complete destruction of all cells in the culture was not observed, a titration method depending on a 50% dilution endpoint could not be relied on. In neutralisation tests carried out with undiluted tissue culture virus, however, cytopathic changes were never found when the tissue culture virus was pretreated with hyperimmune rabbit serum prepared against wart virus isolated from human tissues, or wart virus grown in tissue culture, and some human sera from patients with warts. The cytopathic changes were, however, always observed when tissue culture
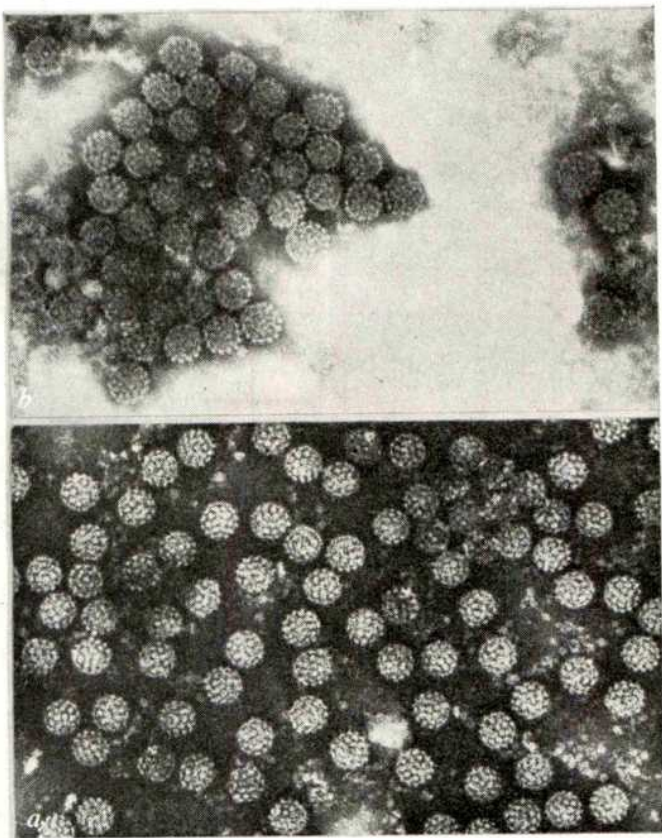
Fig. 2 Electron micrographs of HWV stained with sodium phosphotungstate, *p*H 7.0. *a*, Tissue culture virus from passage 7 (10^7 particles ml^{-1} concentrated from 400 ml supernatant). ×102,600. *b*, Virus extracted from human warts (10^{13} virus particles ml^{-1}). ×102,600. Both represent virions that were banded in CsCl gradient at a density of 1.33 g cm^{-3}. BE tissue culture cells were always infected as a suspension, originally with 10% (w/v) extract of pooled human skin warts (5×10^5 cells were infected with 0.5 ml virus suspension containing 5×10^{12} virus particles). After 2 h incubation and *p*H stress, the cells were plated in a 60 ml Petri dish and 4.5 ml of medium added. As cytopathic changes occurred at first rather rapidly, the cells and supernatant were collected after only 5 d. Cells suspended in the supernatant were freeze–thawed three times and then pelleted at 800 r.p.m. for 10 min. The pellet was hand-homogenised in 1 ml original supernatant and the whole sample was centrifuged at 800 r.p.m. for 10 min to remove debris. Final supernatant was then considered as virus stock at passage 0. (The original inoculum was therefore diluted 1:10 and if no virus was lost, its concentration could be 5×10^{11} ml^{-1}.) Fresh cells were treated in the same way as before (but 2 ml of virus stock used for 1×10^6 cells) and the cells plated into 20 ounce plastic bottles diluting the cells in 20 ml medium. Many rounded cells appeared first after 5 d, when the medium was withdrawn and replaced with 20 ml fresh medium. Cultures were then incubated until more pronounced cytopathic changes were observed (up to 16 d) when they were processed as described for passage 0. The supernatant removed after 5 d was not used for further passage in this set of experiments. The procedure was then repeated at each virus passage. (Each virus passage therefore involves a 1:200 dilution factor. Thus, the original inoculum can be expected to be diluted out by passage 5–6.)
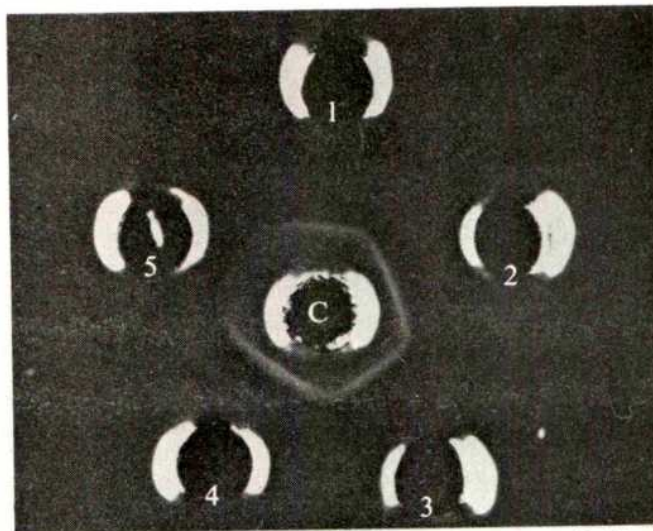
Fig. 3 Double immunodiffusion in 0.7% agarose gel. The centre well (C) contains purified HWV isolated from human wart tissue. The peripheral wells 1, 2, 3 and 4 contain rabbit antisera after two, three and four immunisations, respectively, with purified HWV isolated from the wart tissue. Well 5 was filled with rabbit antiserum after three immunisations with purified HWV grown in tissue culture. Purified viral pellet was resuspended in a small volume of Tris–EDTA buffer, *p*H 7.4, and sonicated for 60 s with a Biosonic sonicator. This was used as antigen for immunodiffusion studies. Rabbits were immunised with purified virus, emulsified in an equal volume of complete Freund's adjuvant and given subcutaneously in multiple sites. Four weeks later, the same amount of antigen in incomplete Freund's adjuvant was similarly injected. Subsequent immunisations were started 3 weeks later and given intravenously at 10 d intervals.

virus was similarly treated with foetal calf serum, preimmune rabbit sera, or sera from healthy blood donors.

To show that the virus was actively multiplying in culture and was not merely being carried along passively from culture to culture, we attempted to demonstrate incorporation of ³H-thymidine into newly formed virions (Fig. 1).

Density gradient centrifugation of labelled virions in a CsCl gradient resulted in a split peak of radioactivity at buoyant density approximately 1.31 and 1.33 g cm^{-3} as well as another peak at a density of 1.39 g cm^{-3}. The 1.31–1.33 peak contained typical wart virus particles (Fig. 2*a*), whereas no virus was found in the peak corresponding to $\rho = 1.39$ g cm^{-3}. Interestingly, the later peak contained mainly hexagonal structures with a dense interior, suggesting that they may represent viral cores. Heavier material sedimenting to the bottom of the tube remains to be investigated.

Gradient centrifugation of unlabelled virions, derived from tissue culture as well as from human wart extracts, usually showed three visible bands, two at the above described densities and a third at a density of approximately 1.29 g cm^{-3} which contains mainly empty particles. These findings are consistent with the observations of Pass and Maizel[13] who found three visible viral bands at similar densities in virus preparations made from human plantar warts.

The tissue culture grown virus was identified as HWV by morphological and immunological analyses. The particles shown in Fig. 2*a* are identical in size and morphology to those of the viruses isolated from human warts (Fig. 2*b*). The average diameter of these two groups of particles is 532 Å, a value which is larger than the size (430 Å) of the polyoma SV40 group of papovaviruses. Thus, by virtue of its larger size[14,15], the particles observed in this study may be distinguished from SV40 viruses.

Antisera made against the virus isolated from human warts, as well as antisera made against the virus propagated in tissue culture, gave a line of identity in immunodiffusion when reacted against purified virus from human warts (Fig. 3). Similar results were obtained with virus grown in tissue culture.

A second line of precipitation was occasionally seen in these immunodiffusion tests. None of these sera, however, reacted with SV40 viral antigens. Lack of cross-reactivity was also seen by immunofluorescence when SV40 infected cells were tested against anti-HWV serum and vice versa.

These observations strongly suggest that HWV has been propagated in tissue culture. This study may provide means for: further studies on the identity of viruses causing human warts, venereal warts and laryngeal papillomas[16,17]; and comparing the properties of HWV with those of other human papovavirus isolates[18-20]. Moreover, as warts are common complications in patients with primary and secondary immuno-deficiencies and in patients under immunosuppression[21], we believe that a system for the study of HWV *in vitro* may provide tools for the study of immune mechanisms responsible for the defence against virally-induced tumour in man.

We thank Drs N. Orentreich and B. Safai for human wart tissue, Dr T. Takahashi for help with immunodiffusion, and Drs F. Kingsley Sanders, P. Gomatos and C. Lopez for discussions and critical reading of the manuscript. This research was supported by the US Public Health Service and the National Cancer Institute.

MAGDALENA EISINGER
OLGA KUCAROVA
NURUL H. SARKAR
ROBERT A. GOOD

Memorial Sloan-Kettering Cancer Center,
1275 York Avenue,
New York, New York 10021

1 Melnick, J. L., *Science*, 138, 1128-1130 (1962).
2 Almeida, J. D., Howatson, A. F., and Williams, M. G., *J. Invest. Dermat.*, 38, 337-345 (1962).
3 Boyle, W. F., Riggs, J. L., Oshiro, L. S., and Lennette, E. H., *Laryngoscope*, 83, 1102-1108 (1973).
4 Oirel, J. D., and Almeida, J. D., *Br. J. vener. Dis.*, 46, 37-42 (1970).
5 Ruiter, M., and Van Mullem, P. J., *J. Invest. Dermat.*, 47, 247-250 (1966).
6 Variot, G., *J. clin. Therap. Infant.*, 2, 529-531 (1894).
7 Ullman, E. V., *Acta Otolaryng.*, 5, 317-334 (1923).
8 Waelsch, L., *Archs Dermat. Syph.*, 124, 625-646 (1917).
9 Rosenbaum, H. D., Alavi, S. M., and Bryant, C. R., *Radiology*, 90, 4 (1968).
10 Rowson, K. E. K., and Mahy, B. W., *Bact. Rev.*, 11, 110-131 (1967).
11 Murray, R. F., Hubbs, J., and Payne, B., *Nature*, 232, 50 (1971).
12 Pass, F., and Marcus, D. M., *J. Invest. Dermat.*, 60, 301-306 (1973).
13 Pass, F., and Maizel, J. V., *J. Invest. Dermat.*, 60, 307-311 (1973).
14 Grawford, L. V., and Grawford, E. M., *Virology*, 21, 258-263 (1963).
15 Black, P. H., Grawford, E. M., and Grawford, L. V., *Virology*, 24, 381-387 (1964).
16 Ogilvie, M. M., *J. Hyg.*, 68, 479-490 (1970).
17 Almeida, F. D., Oriel, J. D., and Stannard, L. M., *Microbios*, 1, 225-232 (1969)
18 Gardner, S. D., Coleman, D. V., Field, A. N., nad Hulme, B., *Lancet*, i, 1253-1257 (1971).
19 Padgett, B. L., Zu Rhein, G., Walker, D. L., and Eckroade, R. J., *Lancet*, i, 1257-1260 (1971).
20 Takemoto, K. K., *et al.*, *J. natn. Cancer Inst.*, 53, 1205-1207 (1974).
21 Spencer, E. S., and Anderson, H. K., *Br. med. J.*, 3, 251-254 (1970).

Persistence of viral DNA in human cell cultures infected with human papilloma virus

THE human papilloma (wart) virus produces a transmissible, benign neoplastic disease which is self-limiting and often regresses. Although rare, papillomas containing papovavirus-like particles may undergo malignant transformation. Papil-lomas in epidermodysplasia verruciformis and condyloma acuminatum can become neoplastic[1-4]. Reports of the wart virus replicating in or transforming cells in culture, however, have not been substantiated[5-7]. Butel was unable to demon-strate cytopathic effect, the production of viral antigens or virus replication using a variety of cell lines and culture tech-niques[8]. Mild stimulation of cellular DNA synthesis, however, was observed in contact-inhibited embryonic human kidney cells after infection[8]. We also have been able to induce cellular DNA synthesis in confluent monolayers of a cell line derived from foetal human foreskin (NHP cells) following infection by wart virus.

Induction of DNA synthesis by papovaviruses may be essential for integration of viral DNA[9-12] and thus it is possible that during induction of cellular DNA synthesis by the wart virus, viral DNA was integrated into host DNA. Such genetic
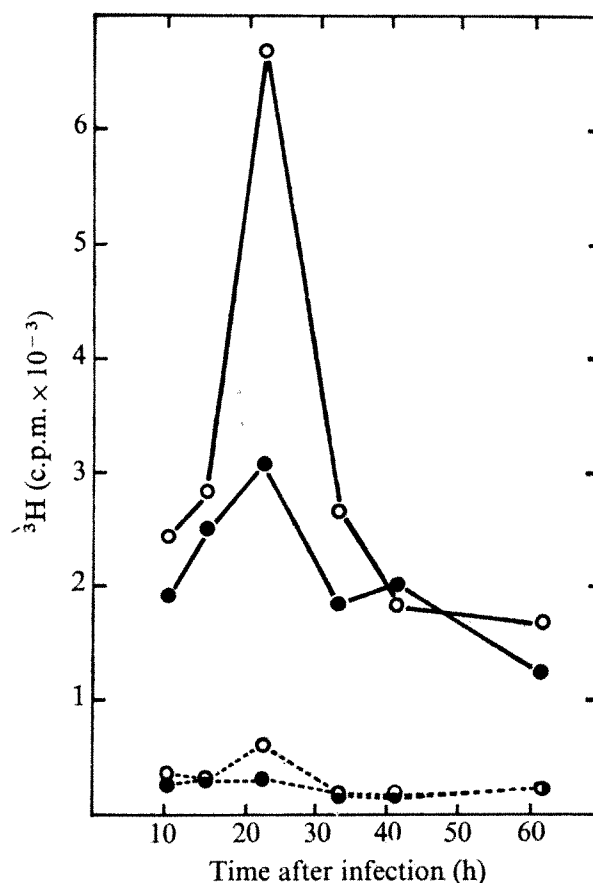
Fig. 1 Induction of cellular DNA synthesis by the human wart virus. Monolayers of NHP cells were grown in 15×60 mm dishes in Eagle's medium containing 10% foetal calf serum at 36 °C. When cell cultures were confluent, the temperature was decreased to 33 °C for 12 h. Duplicate cultures were washed with medium and either infected with 0.2 ml virus or overlaid with 0.2 ml medium for 1.5 h at 33 °C. Cultures were then overlaid with 5 ml medium containing 2% foetal calf serum and maintained at 33 °C. At various times after infection, cultures were labelled for 3 h with 1.0 ml Eagle's medium con-taining 5% dialysed horse serum and 10 μCi ml^{-1} ^{3}H-thymidine (6.7 Ci $mmol^{-1}$, New England Nuclear) followed by a 1 h chase with Eagle's medium containing 10% calf serum. Plates were washed twice with cold phosphate-buffered saline[15], cells lysed and fractions separated according to the procedure of Hirt[16]. Hirt pellets and a portion of the Hirt supernatant fluid were made 1 N with 10 N NaOH and incubated for 60 min at 50 °C. Triplicate samples (0.025 ml) were spotted on filter paper disks (Schleicher and Schuell, 740E) and treated for TCA-precipitable materials using a batch technique[17]. Filter paper disks were counted in toluene scintillation fluid. ○——○, Infected pellet; ○– – –○, infected supernatant; ●——●, control pellet; ●– – –●, control supernatant.

information was detected using wart viral DNA labelled *in vitro* as a probe in DNA-DNA hybridisation studies. We found wart viral DNA persisting in cells 11 months after infection.

Virus was isolated from 5 g tissue homogenised in a Virtis at 45,000 r.p.m. at 4 °C for 30 min in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl followed by a second homo-genisation (10,000 r.p.m., 4 °C, 5 min) in an equal volume of 1,1,2-trichlorotrifluoroethane[13] (freon). The aqueous phase containing virus was separated from the freon phase and virus pelleted at $54,000g$ for 4 h at 4 °C. Pellets were suspended in CsCl ($\rho = 1.33$ g ml^{-1} in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA) and centrifuged to equilibrium at 35,000 r.p.m. at 25 °C in the SW50 rotor. DNA was isolated following rupture of virions with 1% sodium dodecyl sulphate (50 °C, 15 min) and by extraction with phenol. DNA was precipitated with ethanol and supercoiled molecules isolated by CsCl-EtBr equilibrium centrifugation[14].

Induction of cellular DNA synthesis by the wart virus is shown in Fig. 1. Twenty-four hours after infection there was a

Table 1 Summary of the reassociation of ^{32}P-labelled wart viral DNA in the presence of unlabelled cellular DNA

Experiment	DNA source	Cellular DNA (mg ml⁻¹)	^{32}P-wart DNA (ng ml⁻¹)	Factor of increased rate*	No. of unlabelled viral genome equivalents per diploid cell†
1	NHP	1.6	0.8	—	—
	B. subtilis	1.6	0.8	—	—
	Infected NHP	1.6	0.8	1.4	0.16
	B. subtilis+ 2.0 genome equivalent unlabelled wart viral DNA per diploid cell	1.6	0.8	6.7	2.28
2	NHP	2.4	0.6	—	—
	Wi38	2.4	0.6	—	—
	Salmon sperm	2.4	0.6	—	—
	Infected NHP	2.4	0.6	2.0	0.20
	Infected Wi38	2.4	0.6	0.0	0.0
	Salmon sperm+ 0.6 genome equivalent unlabelled wart viral DNA per diploid cell	2.4	0.6	3.8	0.56

*Increased rate relative to reassociation of ^{32}P-viral DNA in presence of control cellular DNA.
†Calculated by the method of Gelb *et al.*[25].

t
wofold increase in the amount of ^{3}H-thymidine incorporated into Hirt pellets of infected cultures compared with mock-infected controls, followed by a decrease to control levels by 32.5 h. Similar stimulation was observed in Hirt supernatant fluids. Examination of infected supernatant fluids in CsCl–EtBr equilibrium gradients and by CsCl velocity centrifugation, however, revealed neither superhelical DNA nor DNA with sedimentation rates expected for wart viral DNA.

DNA–DNA reassociation was used to show the presence of viral DNA sequences in infected cells which had been maintained in culture for several months. Cellular DNA was partially purified by the method of Meinke *et al.*[17] and was subsequently treated with RNase (13 μg ml⁻¹, 37 °C, 30 min), then Pronase (25 μg ml⁻¹, 37 °C, 30 min) and phenol extracted. Cellular DNA was then sheared to an average single-stranded piece size of 560 nucleotides[18].

To label wart viral DNA *in vitro*, DNA was first nicked with pancreatic DNase I at a ratio of 1,000:1 (w/w) in 50 mM

Tris-HCl, pH 7.4, 50 mM KCl, 5 mM MgCl₂ for 20 min at 37°C. DNase was inactivated by heating solutions to 70 °C for 10 min. Nicked DNA was labelled in conditions of repair synthesis with *Escherichia coli* DNA polymerase I (refs 19 and 20). Specific activities of ^{32}P-TTP and ^{32}P-TTP + ^{32}P-dATP-labelled DNAs were 1.95×10^7 and 4.85×10^7 c.p.m. per μg DNA, respectively. Labelled DNAs had sedimentation rates in alkaline sucrose gradients of 5.9 and 6.3S. Sedimentation rates did not change during experiments. Reannealing of denatured labelled DNAs were at rates expected for viral DNAs with piece sizes as described[21]. ^{32}P-viral DNA reassociated to 90% in the presence of fivefold excess of nicked unlabelled wart DNA. SV40 and wart DNA have the same guanine+cytosine content (41%)[22,23] and *in vitro* labelled wart DNA had a similar T_m (86.0 °C), as determined by hydroxyapatite chromatography[18], as did *in vivo* labelled SV40 DNA (86.6° C).

The kinetics of DNA–DNA reassociation follows the
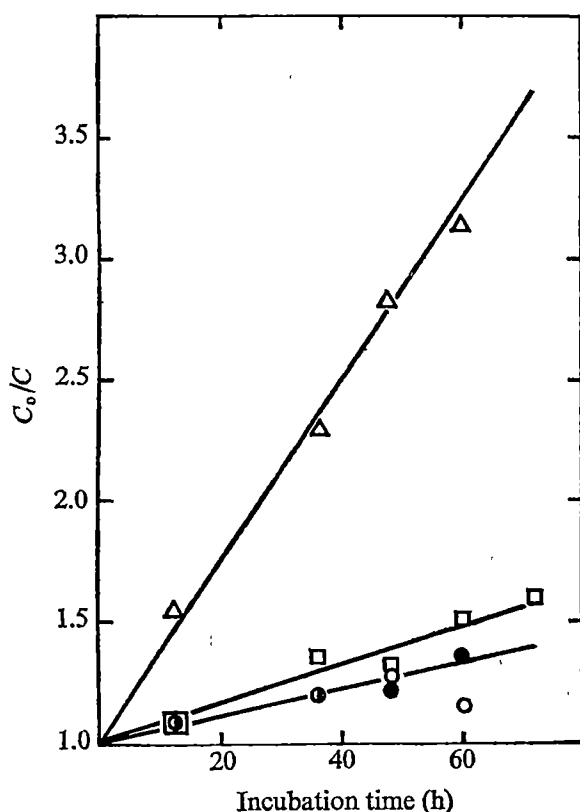
Fig. 2 Reassociation of ^{32}P-labelled wart DNA in the presence of unlabelled DNA from infected NHP cells, mock-infected NHP cells or *B. subtilis* Nicked viral DNA (1 μg) was suspended in a volume of 1.0 ml containing 66 μmol potassium phosphate, pH 7.4, 6.6 μmol MgCl₂, 1 μmol β-mercaptoethanol, 0.05 μmol each of dATP, dGTP, dCTP, and 2 mCi ^{32}P-TTP (114.8 Ci mmol⁻¹) or 0.05 μmol each of dGTP and dCTP with 2 mCi each ^{32}P-TTP and ^{32}P-dATP (118.0 Ci mmol⁻¹, New England Nuclear). DNA repair synthesis was initiated by addition of 20 units *E. coli* DNA polymerase I (Grand Island Biological) for 75 min at 15°C and terminated with 1% Sarkosyl. Labelled DNA was separated from low molecular weight material by chromatography on Sephadex G-50 equilibrated with 0.1% Sarkosyl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA and deproteinated by phenol extraction. Solutions of cellular DNA (1.6 mg ml⁻¹) and ^{32}P-labelled viral DNA (0.8 ng ml⁻¹) were denatured in 0.48 M sodium phosphate, pH 6.8, 0.05% SDS, 0.001 M EDTA at 109 °C for 5 min in screw-capped tubes. Solutions of single-stranded DNA were then allowed to reassociate at 64 °C. At various times samples were removed and diluted to 2.0 ml in 0.14 M sodium phosphate, pH 6.8, 0.05% SDS. Single-stranded DNA was separated from reassociated double-stranded DNA by hydroxyapatite column chromatography (10 × 15 mm) at 60 °C[24]. Single-stranded DNA was eluted from the column with five 2.0 ml washes of 0.14 M sodium phosphate, pH 6.8, 0.05% SDS; reassociated double-stranded DNA eluted with four 2.0 ml washes of 0.48 M sodium phosphate, pH 6.8, 0.05% SDS. Radioactivity was determined for each wash by addition of 15 ml toluene–Triton X-100 (2:1 v/v) scintillation fluid containing Cab-O-Sil (Packard). ○, Mock-infected NHP cellular DNA; □, infected NHP cellular DNA; ●, *B. subtilis* DNA; △, *B. subtilis* DNA with 4.0 ng ml⁻¹ unlabelled nicked wart viral DNA. Each point represents about 500 c.p.m.

equation $C/C_0=1/(1+KC_0t)^4$ (ref. 24); C_0 is the total ^{32}P-viral DNA concentration, C is the concentration of single-stranded ^{32}P-viral DNA, t is time, and K is the reassociation constant. A plot of C_0/C against t results in a straight line with the slope proportional to C_0. The rates of reassociation of ^{32}P-labelled wart DNA in the presence of either *Bacillus subtilis* DNA or mock-infected NHP cellular DNA were very similar, indicating that there is no homology between NHP cellular DNA and wart viral DNA (Fig. 2). Addition of 4.0 ng ml^{-1} unlabelled nicked wart DNA (2.0 copies viral DNA per diploid cell) to a solution of ^{32}P-labelled wart DNA (0.8 ng ml^{-1}) and *B. subtilis* DNA increased the rate of reassociation of ^{32}P-labelled wart DNA by a factor of 6.7, which is in agreement with the expected value of 6.0. When denatured ^{32}P-labelled viral DNA was allowed to reassociate in the presence of DNA derived from infected NHP cells, the rate of reassociation was 1.4 times greater than controls. In another experiment, control cellular DNAs consisted of salmon sperm, human lung (Wi38) and NHP. The rate of reassociation of ^{32}P-labelled wart DNA was similar in each instance. The rate of reannealing of ^{32}P-labelled viral DNA in the presence of infected NHP cellular DNA, however, was twofold greater than controls. Interestingly, no wart viral DNA sequences could be detected in Wi38 cells infected in the same manner as NHP cells. The results are summarised in Table 1. The increases in the reassociation rates correspond to 0.18 wart viral genome equivalents per infected NHP cell.

The increase in the reassociation rate could not be the result of the presence of virus inoculum as input virus would have been diluted beyond detection by cell replication by the methods used. Cells used in these experiments had undergone 15 to 60 doublings after infection. The maximum possible multiplicity of infection as determined by particle counts was about 100 virions per cell. After 15 doublings, there would only be 0.003 viral genome equivalents per cell, which is well below the values detected. The stable persistence of viral genetic information in infected cells must, therefore, be the result of replication of viral DNA sequences which may be integrated into the host genome.

As infected NHP cells were not cloned, detection of 0.18 wart viral genome equivalents per diploid cell indicates that each cell contains a portion of the viral genome or that only some cells carry a complete genome. It is possible that cells carrying the viral DNA have no selective advantage over that of uninfected cells in the culture and thus remain in relatively low numbers.

SV40 DNA can persist in abortively transformed mouse cells[26] and recently, it was shown that SV40 DNA injected into the blastocoel of mouse blastocysts resulted in persistence of SV40 DNA sequences through subsequent maturation to adult mice[20]. Our results indicate a similar situation may exist for the human wart virus in that there is a persistence of wart gene sequences in infected NHP cells.

As the human wart virus is the only known tumour agent of man, it is of interest that wart viral DNA can persist in a cryptic state in infected NHP cells *in vitro*. Although cells seem to be normal with respect to growth and morphology the possibility exists that under proper conditions the latent viral DNA sequences could become 'derepressed' and induce cell growth characteristic of transformed cells. These results would indicate that the ubiquitous human wart virus should be given serious consideration as a possible agent of neoplastic disease in man.

WAYNE D. LANCASTER
WILLIAM MEINKE

Department of Microbiology,
Scripps Clinic and Research Foundation,
La Jolla, California 92037

1 Aaronson, C. M., and Lutzner, M A , *J Am med Assoc.*, 201, 775–777 (1967).
2 Yabe, Y , Okamoto, T., Ohmori, S , and Tanioku, K , *Dermatologica*, 139, 161–164 (1969)
3 Delescluse, C , *et al* , *Archs Derm. Forsch* , 242, 202–215 (1972)
4 Kovi, J., Tillman, R. L , and Lee, S. M , *Am J. clin. Path* , 61, 702–710 (1974)
5 Rowson, K F. K , and Mahy, B W. J , *Bact. Rev* , 31, 110–131 (1967)
6 Oroszlan, S., and Rich, M A , *Science*, 146, 531–533 (1964).
7 Noyes, W. F., *Virology*, 25, 358–363 (1965).
8 Butel, J S., *J natn Cancer Inst.*, 48, 285–293 (1972)
9 Dulbecco, R., Hartwell, C H , and Vogt, M., *Proc. natn Acad. Sci U S.A* , 53, 403–410 (1965).
10 Sheinen, R , *Virology*, 29, 167–170 (1966).
11 Kit, S., de Torres, R A , Dubbs, D R., and Selvi, M L , *J. Virol* , 1, 738–746 (1967)
12 Lehman, J. M., and Defendi, V., *J Virol.*, 6, 738–749 (1970).
13 Girardi, A. J , *Virology*, 9, 488–489 (1969).
14 Radloff, R., Bauer, W., and Vinograd, J , *Proc. natn. Acad Sci U.S.A.*, 57, 1514–1521 (1967)
15 Dulbecco, R., and Vogt , M., *J exp Med* , 99, 167–182 (1954)
16 Hirt, B , *J molec Biol* , 26, 365–369 (1967)
17 Meinke, W , Goldstein, D. A , and Hall, M. R , *Analyt Biochem* , 52, 82–88 (1974).
18 Meinke, W., and Goldstein, D A , *J molec Biol* , 86, 757–773 (1974).
19 Nonoyama, M , and Pagano, J S , *Nature*, 242, 44–47 (1973).
20 Jaenisch, R., and Mintz, B , *Proc. natn Acad Sci. U S A* , 71, 1250–1254 (1974).
21 Wetmur, J G , and Davidson, N., *J. molec Biol.*, 31, 349–370 (1968)
22 Crawford, L V., and Crawford, E. M , *Virology*, 21, 258–263 (1963)
23 Crawford, L V , and Black, P. H., *Virology*, 24, 388–392 (1964)
24 Britten, R J., and Kohne, D. E , *Science*, 161, 529–540 (1968).
25 Gelb, L D., Kohne, D E., and Martin, M A.,*J. molec. Biol.*, 57, 129–145 (1971)
26 Smith, H S , Gelb, L D , and Martin, M. A , *Proc natn Acad Sci. U.S.A* , 69, 152–156 (1972)

Effect of bromodeoxyuridine on redundancy of ribosomal RNA cistrons of *Drosophila virilis*

THERE have been numerous demonstrations that the thymidine analogue, 5-bromodeoxyuridine (BrdU), can affect the progress of cellular events in eukaryotes It has been suggested that the incorporation of the base analogue changes the secondary structure of DNA and/or chromatin recognition sites[1-3]. Support for this view has come from studies which have shown a BrdU substitution effect on the physical properties of isolated chromatin[4,5] and alterations in the patterns of RNA transcription[6-9]. In some instances, a correlation between the onset of DNA synthesis and the effect of BrdU has been observed[1,3]. In addition, several studies have shown that the drug can alter the process of DNA replication[8,10,11]. Thus, it has been argued that changes in the replication of DNA may precede or lead directly to the expression of BrdU sensitivity

We have initiated a study of the influence of BrdU on the redundancy of the ribosomal RNA (rRNA) cistrons of *Drosophila*. The rDNAs have been localised to the nucleolus organiser regions of the *Drosophila* karyotype[12], and can be manipulated genetically. The redundancy of the rRNA cistrons can be measured by hybridisation techniques and evidence of mechanisms which control both the replication[13] and transcription[14,15] of these sequences has been presented. Thus, the interaction of transcriptional and replicational influences of BrdU can be examined with regard to the behaviour of a single DNA sequence. In this report we describe an effect of BrdU on the redundancy of rRNA cistrons of *D. virilis*. *D. virilis* was chosen because a substantial portion of the diploid genome contains A–T-rich redundant sequences. These sequences have been localised to the heterochromatic regions of the *D. virilis* karyotype[16] which also contain the rRNA cistrons It was argued that A–T-rich regions may preferentially incorporate BrdU

BrdU was added to the medium of *D. virilis* larvae on days 5 and 7 after the parental adults had been placed on the food The redundancy of the rRNA cistrons in DNA from the BrdU-treated larval material did not differ significantly from that of the untreated larval control populations (Table 1, experiment 1) When drug-treated larvae from this experimental population were reared to the adult stage, however, a substantially smaller fraction of the DNA hybridised with rRNA compared with controls. Note that the rRNA cistron redundancy is repeatedly

Fig. 1 Thermal stability of ^{3}H-rRNA–DNA hybrids. Denatured DNA from *D. melanogaster*, *D. virilis*, and BrdU-treated *D. virilis* (days 5 and 7, experiment 1, Table 1) adults was loaded on to filters and the hybridisation reactions performed as described in the legend to Table 1. After RNase treatments the filters were washed and placed in glass vials with 3 ml 1 × SSC and incubated in a water bath. Starting at 45 °C, the temperature was raised in 5° increments and maintained for 5 min before removing the solution and replacing it with fresh SSC at the same temperature. Counts released from the filters were determined using Aquasol (New England Nuclear). *D. melanogaster* ^{3}H-rRNA hybrids formed with *D. melanogaster* DNA (△); *D. virilis* adult DNA (●); BrdU-treated (days 5 and 7) *D. virilis* adults (○).

found to be lower in DNA from larval tissues than DNA from adult tissues of *D. virilis*. Similar results have been reported for other species of *Drosophila*[21]. The observation was anticipated because the rDNAs are underreplicated in polytene cells[7,13], and these cells are most prominent in the larval stages.

When the experiments were repeated, two populations were established for drug treatment. In one case, drug was spread on the food on days 5 and 7 after the parental adults began egg laying, as in the previous experiment. Drug was administered to the second group on days 2 and 5, after egg laying had indicated whether the BrdU effect was limited to the postlarval stages. The results obtained from treatments on days 5 and 7 (Table 1, experiment 2) agree with those of experiment 1. There was no substantial difference in rDNA content between larval BrdU-treated and control DNAs, but a significant decrease in the rDNAs from treatments on days 5 and 7 (Table 1, experiment 2) difference in rDNA content between larval BrdU-treated ments 1 and 2 probably reflects variations in the number of larvae grown on the food and the time over which the drug was available to the larvae. DNA extracted from larvae treated with BrdU on days 2 and 5 (Table 1) contained significantly lower rDNA redundancy (18%) than control larvae. DNA from the adults of this population, however, showed about one-half the effect (~9%) compared with DNA from untreated adults. Thus, BrdU influences the redundancy of the rRNA cistrons in both

adult and larval tissues. In the conditions used, however, the effect, if prominent in larval material, was not entirely sustained into the adult tissues.

Note that we have made no effort to screen for any of the development aberrations reported by other workers using BrdU on developing *Drosophila* systems[17,18]. BrdU-treated adults are not phenotypically bobbed The major gross effect of our BrdU treatments on larvae is an increase in the time required to reach adult eclosure. Viability, fertility, and sex ratios of eclosing BrdU-treated adults are not significantly affected by these experimental conditions (D. Durica and H.M.K., unpublished).

Two controls were performed with regard to the artefacts which BrdU might cause in the measurement of hybrids in our heterologous DNA–RNA reactions. In the first, the thermal stability of the hybrids formed between *D. melanogaster* ^{3}H-rRNA and DNA from BrdU-treated and control *D. virilis* adults was compared with homologous *D. melanogaster* hybrids. A typical melting profile from each type of hybrid is presented in Fig. 1, which shows that the thermal stability of the heterologous hybrid is comparable with the stability of homologous *D melanogaster* DNA–RNA complexes. Furthermore, the DNA from BrdU-treated material formed hybrids of comparable quality to those formed by the other DNAs. These thermal stability studies, however, were performed on RNase-resistant hybrids. Thus, it might be argued that BrdU and control *D. virilis* DNAs have the same number of rRNA cistrons, but the hybrids formed by DNAs from BrdU-treated material are more sensitive to RNase. To examine this possibility, the radioactivity (^{3}H) associated with filters was determined before and after RNase treatment for DNAs from BrdU-treated adults (Table 1, experiment 2) and controls. There was no difference in RNase sensitivity of the DNA–RNA hybrids formed by these DNA samples. Thus, the hybrids formed by DNA from BrdU-treated material seem to be neither less stable nor more

Table 1 Hybridisation of *D. melanogaster* ^{3}H-rRNA to DNA of BrdU-treated *D. virilis*

| | Day of BrdU addition | % DNA in hybrid | | % Change |
		Experiment 1	Experiment 2	
Larval	2 and 5	—	0.290	18.3
	5 and 7	0.356	0.351	0–1.1
	Control	0.355	0.355	—
Adult	2 and 5	—	0.352	9.5
	5 and 7	0.212	0.271	45 5–30.3
	Control	0.389	0.389	—

D. virilis were grown on standard corn meal, agar, and sucrose media at 22 °C in half-pint bottles. Adults were placed on fresh media for 24 h, the bottles were then cleared, and at various times 2 ml 10^{-3} M BrdU solution (Sigma) were spread on top of the media. This concentration and means of application was chosen on the basis of other BrdU studies in developing *Drosophila* tissues[17,18], and some preliminary observation communicated by Juan Valencia (University of Wisconsin, Madison). Larvae in the third instar were collected by flotation away from the food with 20% sucrose and washed extensively in distilled water. DNA extractions from larvae and adults were performed as described elsewhere[19]. Nitrocellulose filters loaded with about 20 μg denatured DNA were incubated for 12 h at 67 °C in 4 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% sodium lauryl sulphate, and 3.75 μg ml⁻¹ (68,800 c p.m. μg⁻¹) ^{3}H-rRNA. ^{3}H-rRNA was prepared from *D. melanogaster* cell lines using the technique of Brown and Littna[20] (gift of Dr L. Weber, SUNY, Albany). The filters were washed with 2 × SSC, treated with 20 μg ml⁻¹ RNase A at 25 °C for 2 h, washed again with 2 × SSC, and counted in Liquifluor (New England Nuclear) after drying. Scintillants were removed from the filters by several washings with toluene and ether, and the filter-bound DNA was determined using the procedure of Kissane and Robbins[26]. All DNA preparations were tested in the same reaction vessel. A minimum of six measurements was made for each type of DNA. Untreated larval and adult DNA preparations were used as internal standards to compare hybridisations of experiments 1 and 2. Maximum s.e.; experiment 1, ±0.022; experiment 2, ±0.013.

RNase-sensitive than the hybrids formed by DNA from untreated tissues. Therefore, the differences measured in the hybridisation reactions must reflect changes in the redundancy of the rRNA cistrons associated with the presence of BrdU in the developing larvae.

We may only speculate as to the mechanism(s) which produce this BrdU effect. The redundancy of the rRNA cistrons seems to be subject to control. The number of rounds of DNA synthesis which the rDNA sequences undergo differs significantly from other nucleotide sequences in polytene chromosomes[7,13,22]. Further, the cells of *D. melanogaster* which bear a substantial deletion of the rDNAs when diploid, possess nearly wild-type levels of rRNA cistrons in polytene tissue[13]. In addition, alteration in rDNA redundancy of non-polytene cells can occur in *D. melanogaster*[23]. It has been suggested that such rDNA magnifications occur through unequal exchange events between sister chromatids before formation of meiocytes[24]. The presence of BrdU in developing larvae may affect any of the events normally associated with establishing and maintaining the redundancy of rRNA cistrons in polytene and diploid cells. To date we have no direct evidence that the BrdU effect described here is limited to particular tissues or cell types; nor is it clear that the effect is limited to rRNA cistrons. Preferential incorporation of BrdU into highly and intermediately repeated DNA sequences has been observed in tissue culture cells grown at low drug concentrations[6,25]. Such preferential incorporation may explain the specificity of the action of BrdU in this case, and further suggest that the A–T-rich redundant sequences which surround the rDNA may also be influenced by the drug. Experiments designed to examine some of these possibilities are in progress.

This work has been supported by the University of Connecticut Research Foundation, the National Science Foundation, and the National Cancer Institute.

H. M. KRIDER

Biology Division,
University of Connecticut,
Storrs, Connecticut 06268

J. A. BLAKE

Biological Laboratories,
Harvard University,
Cambridge, Massachusetts 02138

1 O'Neill, M. C., and Stockdale, F. E., *Devl Biol.*, 37, 117–132 (1974)
2 Tomida, M., Koyama, H , and Ono, T , *Biochim. biophys. Acta*, 338, 352–363 (1974)
3 Walther, B T., Pictet, R. L., David, J. D., and Rutter, W J , *J. biol. Chem*, 249, 1953–1964 (1974).
4 David, J., Gordon, J. S., and Rutter, W. J., *Proc. natn Acad. Sci. U.S A*, 71, 2808–2812 (1974).
5 Lapeyre, J -N , and Bekhor, I , *J. molec Biol.*, 89, 137–162 (1974).
6 Grady, L. J., and Campbell, W. P., *Expl Cell Res.*, 87, 127–131 (1974)
7 Hennig, W., and Meer, B., *Nature new Biol.*, 233, 70–72 (1971).
8 Kotzin, B. L., and Baker, R. F., *J. Cell Biol* , 55, 74–81 (1972).
9 Preisler, H. D., Housman, D., Scher, W , and Friend, C , *Proc. natn. Acad Sci U.S.A.*, 70, 2956–2959 (1973).
10 Baker, R. F., and Case, S T., *Nature*, 249, 350–352 (1974)
11 Lykkesfeldt, A. E., and Andersen, H. A., *J Cell Biol.*, 62, 316–321 (1974)
12 Ritossa, F., and Spiegelman, S., *Proc. natn. Acad Sci* , *U.S.A.*, 53, 737–745 (1965).
13 Spear, B. B., and Gall, J. G., *Proc. natn. Acad Sci. U.S.A* , 70, 1359–1363 (1973).
14 Krider, H. M., and Plaut, W , *J. Cell Sci* , 11, 675–687 (1972),
  Krider, H. M., and Plaut, W , *J. Cell Sci.*, 11, 689–697 (1972).
15 Mohan, J., and Ritossa, F. M., *Devl Biol.*, 22, 495–512 (1970).
16 Gall, J. G., Cohen, E H., and Polan, M L., *Chromosoma*, 33, 319–344 (1971).
17 Gilbert, E. F , Pitot, H. C , Bruyer, H. J., Jr, and Cheung, A. L., *Teratology*, 7, 205–207 (1973).
18 Rizki, T. M., Rizki, R. M , and Douthit, H A., *Biochem. Genet.*, 6, 83–97 (1972).
19 Weber, L., thesis, Univ Connecticut (1974).
20 Brown, D. D., and Littna, E., *J. molec. Biol.*, 8, 669–687 (1974).
21 Sibatani, A., *Molec. gen. Genet.*, 114, 177–180 (1972).
22 Dickson, E , Boyd, J B., and Laird, C. D., *J. molec. Biol.*, 61, 615–627 (1971).
23 Ritossa, F. M., *Proc. natn. Acad. Sci. U S A.*, 60, 509–516 (1968).
24 Tartof, K. D., *Proc. natn. Acad. Sci. U.S.A.*, 71(4), 1272–1276 (1974)
25 Schwartz, S. A., and Kirsten, W. H., *Proc natn. Acad Sci. U.S.A* , 71, 3570–3574 (1974).
26 Kissane, J. M., and Robins, E., *J. biol. Chem* , 233, 184–188 (1958).

Inhibition of myogenesis in a rat myoblast line by 5-bromodeoxyuridine

5-BROMODEOXYURIDINE (BrdU) at very low concentrations inhibits the expression of cell or tissue-specific characteristics of several differentiating systems (for reviews see refs 1 and 2) including myoblasts[3-6], and was postulated to cause its anti-differentiation effects by being incorporated into DNA segments[2-6]. Available data show, however, that in some cell types, the primary effects of BrdU may be mediated by means other than its incorporation with the genome[7,8]. To determine whether template modification by BrdU *per se* is the cause of inhibition of myogenesis, we have investigated the reversal of inhibition by BrdU by various naturally occurring nucleosides, and show that deoxycytidine (and deoxyuridine) reverses the inhibition of myogenesis caused by BrdU without affecting its incorporation into cellular DNA. It seems likely, therefore, that template modification by BrdU may not be the sole cause of the inhibition of fusion of myoblasts brought about by this compound.

A clone, L6B, which was isolated from Yaffe's[9] rat skeletal muscle myoblast cell line, L6, and which was stable with regard to its fusion properties, was used throughout. Concentrations of BrdU higher than 1 μM during growth and division of the myoblasts completely abolish their differentiation into myotubes (Fig. 1). Below 1 μM BrdU has very little effect on myogenesis. As with the chick myoblasts[5] and other cell types[10], L6B cells reach confluence in the presence of 3.5–16 μM BrdU but are very much flattened. The doubling time and plating efficiency of L6B myoblasts remains unaltered at concentrations of BrdU which completely inhibit fusion (Fig. 1a). The inhibition of differentiation by BrdU is reversed
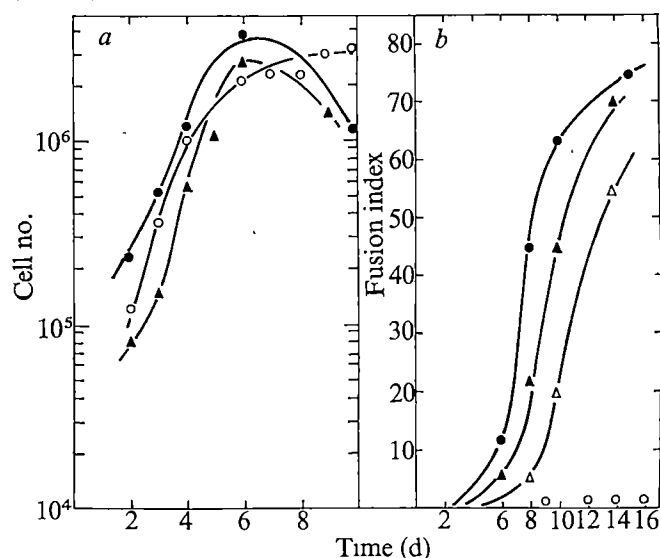
Fig. 1 Effect of BrdU and deoxycytidine on growth (a) and fusion (b) of myoblasts. a, About 10[5] cells of clone L6B were grown in 250 ml Falcon culture flasks in 20 ml Dulbecco modified Eagle's (DME) medium supplemented with 10% horse serum, 50 μg ml⁻¹ gentamycin and the nucleosides to be tested. The cells were incubated at 37 °C in an atmosphere of 10% CO_2 in air. The culture medium was replaced after the first day of plating and every 4th day thereafter. For growth measurement the cells were dissociated using 0.1% trypsin and counted in a haemocytometer b, To obtain the fusion index cells were plated in 6 cm diameter plastic tissue culture dishes at a density of 3.5×10^{3} cells per cm² in 4 ml DME containing 10% horse serum and the required additives. At the indicated times, cells were washed with citrate saline, fixed with methanol, dried, and stained with Ehrlich's eosin–haemotoxylin. Fusion index was determined according to the procedure of Morris and Cole[17] by counting nuclei in single cells and myotubes in 32 fields (0.084 mm² per field) in early stages of fusion, and in 15–20 fields later A cell was scored as a myotube if it contained three or more nuclei within the same cell. ●, No additions; ○, 3.2 μM BrdU; ▲, 3 2 μM BrdU plus 400 μM deoxycytidine; △, 16 μM BrdU plus 400 μM deoxycytidine.
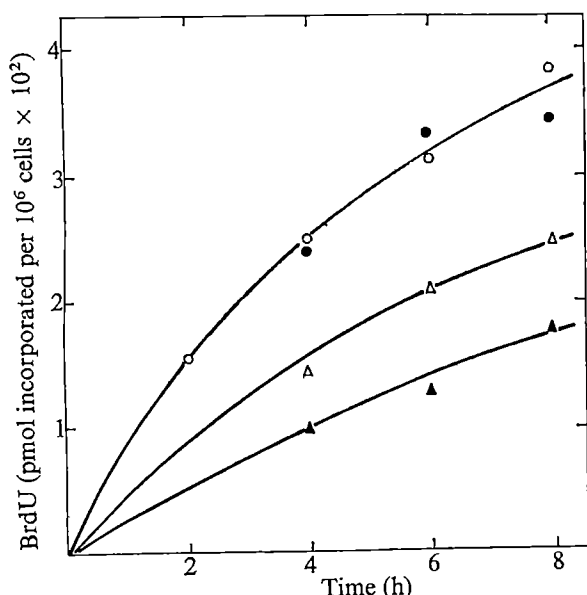
Fig. 2 Effect of nucleosides on the incorporation of BrdU in cells. L6B myoblasts (10⁶ cells) were grown for 3 d in 35 × 10 mm, 6-well Linbro dishes in 5 ml DME containing 10% horse serum. Cells were washed consecutively with phosphate-buffered saline and citrate saline and then exposed to 3 ml growth medium containing 3.2 µM (0.1 µCi) ^{14}C-BrdU (50.6 mCi mmol^{-1}) and other nucleosides at 37 °C. At the indicated intervals the cells were washed twice with saline and then treated for 15 min with 0.5 ml cold, 10% TCA. Cells were once again washed with saline to remove the TCA, dissolved in 0.5 ml 2 N NaOH, and counted. ○, BrdU alone; ●, BrdU plus 0.4 mM deoxycytidine; △, BrdU plus 1 µM thymidine; ▲, BrdU plus 2 µM thymidine.

to a large extent by the presence during growth of deoxy-cytidine, deoxyuridine or thymidine (Table 1).

Tested by themselves or together with BrdU at concentrations listed in Table 1, the nucleosides effective in restoring fusion do not cause any significant alterations in growth and plating efficiency of L6B myoblasts (Fig. 1a). Inhibition caused by higher concentrations of BrdU (16 µM) is also reversed by 400 µM deoxycytidine but the time required for maximal fusion is prolonged (Fig. 1b). Note that not only is the fusion potential of myoblasts restored in the presence of deoxycytidine, but the myotube-specific enzyme, creatine phosphokinase (CPK) also appears and increases in the fused myoblasts. As an example myoblasts in the presence of 3.2 µM BrdU show a specific activity (µmol product formed per min per mg protein) for CPK of less than 0.02, but in myotubes produced in the presence of BrdU and 400 µM deoxycytidine the specific activity rises to about 0.35.

The simplest way to explain the reversal by the nucleosides of BrdU-induced inhibition of differentiation is to assume that they inhibit the entry and subsequent incorporation with DNA of BrdU inside the cells. Steck et al.[11] have demonstrated that

Table 1 Effect of various nucleosides on myoblast differentiation in the presence of BrdU

Addition	Concentration (µM)	Fusion index
None	—	0 2
Deoxycytidine	4	21 ± 2
	40	46 ± 5
	400	46 ± 6
Deoxyuridine	33	31 ± 4
Thymidine	0.5	12 ± 2
	1	52 ± 5
Thymine	1	17 ± 3
Cytidine	400	19 ± 3
Uridine	400	1 ± 0.4
Guanine	400	5 ± 2
Adenine	400	4 ± 2

Cells of clone L6B were plated in the presence of 3 5 µM BrdU and the nucleoside to be tested. Fusion index was quantitated on day 10 after growth. Fusion index in the absence of BrdU was about 50. Details of experimental procedures are given in Fig. 1.

in various cultured cell lines uptake of any given nucleoside is inhibited by other heterologous nucleosides. In L6B myoblasts, however, only thymidine (but not thymine) inhibits the uptake of BrdU into the acid-insoluble material of the cell. We have ascertained that this material is probably DNA because treatment with DNase releases radioactivity in an acid-soluble form. Deoxycytidine (400 µM), however, has no effect on the uptake of BrdU (Fig. 2).

This suggests that thymidine and deoxycytidine reversal may occur through a pathway which is independent of the level of incorporation of BrdU in DNA. To investigate these possibilities further we carried out the following experiments. Myoblasts were grown for one to four generations (in different experiments) in the presence of 3.2 µM ^{14}C-BrdU, 3.2 µM ^{14}C-BrdU plus 1 µM cold thymidine, and 3.2 µM ^{14}C-BrdU plus 0.4 mM deoxycytidine. ^{14}C-BrdU (50.6 mCi mmol^{-1}) was used at a level of 1 µCi in each case. At the end of the required time the cells (about 10⁶) were collected using EDTA, and nuclei were isolated and lysed in 0.5% SDS as described previously[12]. The DNA was banded by centrifugation in neutral CsCl density gradients and the specific activities (sum of c.p.m. in each DNA-containing fraction divided by the sum of A_{260} of the same fractions) computed[12]. The DNA of control cultures (without BrdU or other additions) banded at a density of 1.703 g cm^{-3} and that of cells grown with BrdU (3.2 µM) for two generations at 1.708 g cm^{-3}. The amount of BrdU substitution of DNA was thus not more than 1–2% (ref. 13). The small amount of BrdU incorporated in our experimental conditions may be caused by the presence internally of sufficiently large pools of nucleotides in L6B myoblasts which interfere with the uptake or further metabolism of BrdU.

Whatever the reasons for the low incorporation of BrdU the significant finding was that the specific activity of DNA from cells grown in the presence of 3.2 µM BrdU in different experiments was approximately the same in myoblasts grown without (4.3 × 10⁴), or with 0.4 mM deoxycytidine (4.7 × 10⁴). We conclude that inhibition of fusion of myoblasts and reversal of this inhibition was not dependent on the substitution of thymidine residues in DNA by BrdU. A decrease in the level of BrdU incorporation in the presence of thymidine was found in our experiments (specific activity 3.2 × 10⁴) and was expected in view of the BrdU uptake experiments presented earlier (Fig. 2). It is likely that differentiation of myoblasts is restored in the presence of thymidine (Table 1) either because inhibitory amounts of BrdU are not able to accumulate inside the cells (see later) or thymidine itself is able to counteract, like deoxycytidine, the inhibitory effect of BrdU on the yet unidentified events leading to fusion.

If it is agreed that BrdU substitution in DNA is probably not the cause of inhibition of myogenesis, as the results at least with deoxycytidine show, what are the conceivable mechanisms for BrdU inhibition of fusion? We propose that BrdU in a phosphorylated form at low concentrations inhibits some of the glycosyltransferases of the myoblasts which are responsible for the biosynthesis of fusion-specific glycoproteins or glycolipids and this inhibition is reversed by several pyrimidines related to the sugar donors (UDP-glucose, CMP-N-acetylneuraminic acid, and so on) of macromolecular synthesis. In support of this proposal it may be stated that several nucleoside analogues, such as puromycin[14] and cytosine arabinoside[15] have been shown to inhibit some glycosyltransferases in mammalian cells. In addition, it has been reported[16] that chick embryo fibroblasts grown in the presence of BrdU become agglutinable by concanavalin A, which again suggests that alteration of sugar-containing macromolecules does occur in the presence of BrdU.

JACKILYNN ROGERS
STEPHEN K. C. NG
MARION B. COULTER
B. D. SANWAL

Department of Biochemistry,
University of Western Ontario,
London, Ontario, Canada N6A 5CL

1 Wilt, F H , and Anderson, M., *Devl Biol.*, 28, 443–447 (1972).
2 Rutter, J. T , Pictet, R L , and Morris, P. W., *A. Rev Biochem* , 42, 607–646 (1973)
3 Stockdale, F., Okazaki, K , Nameroff, M , and Holtzer, H., *Science*, 146, 533–535 (1964)
4 Coleman, J. R., Coleman, A. W., and Hartline, E. J. H., *Devl Biol.*, 19, 527–548 (1969)
5 Bischoff, R , and Holtzer, H., *J. Cell Biol.*, 44, 134–150 (1970).
6 O'Neill, M C , and Stockdale, F F., *Devl Biol.*, 37, 117–132 (1974).
7 Schubert, D , and Jacob, F , *Proc. natn. Acad Sci ¦U.S.A* , 67, 247–254 (1970)
8 Meuth, M , and Green, H , *Cell*, 2, 109–112 (1974).
9 Yaffe, D , *Proc. natn. Acad. Sci U S.A* , 61, 477–483 (1968).
10 Littlefield, J W , and Gould, E. A., *J. biol. Chem* , 235, 1129–1133 (1960)
11 Steck, T L., Nakata, Y., and Bader, J. P., *Biochim. biophys. Acta*, 190, 237–249 (1969).
12 Lieberman, M W , and Dipple, A., *Cancer Res.*, 32, 1855–1860 (1972).
13 Rownd, R., *Biochim biophys Acta*, 134, 467–469 (1967)
14 Trelvar, M , Sturgess, J M., and Moscarello, M. A., *J. biol Chem* , 249, 6628–6632 (1974).
15 Hawtrey, A O , Scott-Burden, T , and Robertson, G , *Nature*, 252, 58–60 (1974).
16 Biquard, J. -M., *J. cell Physiol.*, 84, 459–462 (1974)
17 Morris, G. E., and Cole, R. J , *Expl cell. Res* , 75, 191–199 (1972).

Reduction in sympathetic nervous activity as a mechanism for hypotensive effect of propranolol

THE β-adrenoceptor antagonist propranolol is an effective antihypertensive drug in man[1] but it is not known how the fall in blood pressure is produced[2]. There is a decrease in plasma renin activity[3] and in cardiac output immediately on starting propranolol therapy but neither of these effects can explain fully the hypotensive action of the drug which is delayed in onset and associated with a reduction in peripheral vascular resistance[4]. It has been suggested that the fall in blood pressure after propranolol results from blockade of β-like adrenoceptors within the central nervous system[5]. Thus, intracerebroventricular injection of propranolol lowers blood pressure in conscious cats[6] and rabbits[6], an effect which is specific for the β-blocking (—) isomer of the drug[7]. The effective concentration of propranolol achieved in the hypothalamus by this route of administration is within the range predicted for hypertensive patients after chronic oral therapy[8]. We now present direct evidence that propranolol diminishes sympathetic nerve activity in the rabbit and that this central effect contributes to the hypotensive action of the drug.

Electrodes were implanted in the greater splanchnic nerve of rabbits weighing 3–4 kg (ref. 9). After a recovery period of at least 4 d a catheter was inserted under local anaesthesia into the central artery of an ear and used for measurement of blood pressure. Another catheter was inserted into the marginal vein of the other ear for administration of drugs. Each conscious unrestrained animal was placed in an individual cage and left undisturbed for 1 h. Integrated splanchnic nerve activity and arterial pressure were then recorded continuously for a 3-h period. The first hour served for control observations. After these, intravenous infusion of one of four solutions was begun and continued at a rate of 3 ml h^{-1} for the remaining 2 h. The solutions used were saline, (±)-propranolol, (+)-propranolol and sodium nitroprusside, sufficient of each drug being dissolved in saline to enable an infusion rate of 1 mg kg^{-1} h^{-1}. Each treatment was given to eight animals. Splanchnic nerve

Table 1 Effect of intravenous (+)- and (±)-propranolol, sodium nitroprusside and saline on mean arterial pressure (MAP) and splanchnic nerve activity (SNA) in conscious rabbits

Infusion	No. of animals	First hour MAP	First hour SNA	Second hour MAP	Second hour SNA
Saline	8	97±2	111±5	99±3	104±16
(+)-Propranolol	8	101±2	122±17	99±4	132±24
(±)-Propranolol	8	94±3	68±11*	87±2†	52±9*
Sodium nitroprusside	8	74±1†	178±30*	73±2†	199±38*

Values are the mean ±s.e.m. of the average during one hour expressed as a percentage of the average during the hour pretreatment. Significant differences from saline control group: *$P < 0.05$; †$P < 0.01$ (Student's unpaired t test).
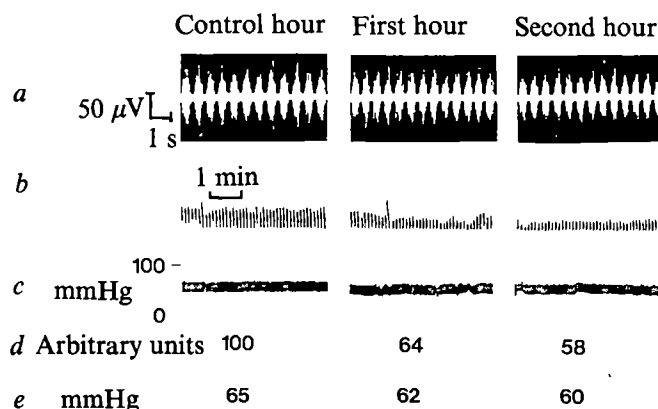
Fig. 1 Effect of (±)-propranolol infusion (1 mg kg^{-1} h^{-1} intravenously) on sympathetic nerve activity and arterial pressure in a conscious rabbit. *a*, Splanchnic nerve discharges as original oscilloscope records and as integrated activity over 6-s periods (*b*); *c*, arterial pressure (each record was taken at the end of the time period indicated); *d*, mean values of integrated splanchnic nerve activity per hour and of mean blood pressure measured over the complete hour by planimetry (*e*).

activity during each hour was summed by planimetry of the record, and the values during the infusion hours expressed as a percentage of that during the pretreatment hour. The systolic and diastolic arterial pressure traces were treated similarly except that the results were expressed in terms of mean arterial pressure, calculated as diastolic pressure plus one third of the pulse pressure.

Table 1 shows the effect of the four treatments on splanchnic nerve activity and mean arterial pressure. Neither saline nor (+)-propranolol, the non-β-blocking isomer of propranolol, altered these parameters significantly. Sodium nitroprusside, a vasodilator drug which relaxes vascular smooth muscle[10], reduced arterial pressure. An increase in preganglionic sympathetic nerve activity accompanied the fall in blood pressure, indicating that the preparation is sensitive enough to detect reflex changes which are the expected response to a peripherally mediated hypotension.

By contrast, a different pattern of response was seen with (±)-propranolol which decreased both blood pressure and splanchnic nerve discharges. Figure 1 shows the original record of such an experiment, illustrating the parallel fall in preganglionic sympathetic nerve activity and blood pressure during (±)-propranolol infusion.

As sympathetic nerve activity was recorded from a preganglionic nerve, the site of the sympatho-inhibitory action of propranolol must be in the central nervous system. The only other possible explanation—that the drug increases the sensitivity of the arterial baroreceptors—seems very unlikely. Therefore, we suggest that blockade of central β-adrenoceptors brings about a reduction in sympathetic nervous tone and that this action could explain the antihypertensive effect of propranolol.

P. J. LEWIS*
G. HAEUSLER

Department of Experimental Medicine of
F. Hoffmann-La Roche & Co., Ltd,
CH-4002 Basle, Switzerland

1 Prichard, B. N. C., and Gillam, P. M. S , *Br Med J* , 1, 7 (1969).
2 Frohlich, E. D., *New Engl. J Med.*, 287, 1247 (1972).
3 Buehler, F. R , Laragh, J. H., Baer, L., Vaughan, E. D , and Brunner, H. R , *New Engl. J. Med.*, 287 1209 (1972).
4 Tarazi, R. C , and Dustan, H. P., *Am J. Cardiol.*, 29, 633 (1972).
5 Day, M D , and Roach, A G., *Nature*, 242, 30 (1973)
6 Lewis, P. J , Reid, J. L., Myers, M. G., and Dollery, C. T , *J. Pharmac. exp. Ther.*, 188, 394 (1974).
7 Day, M. D , and Roach, A. G , *Clin. exp Pharmac. Physiol.*, 1, 333 (1974)
8 Myers, M. G., Lewis, P. J., Reid, J. L., and Dollery, C. T., *J. Pharmac. exp Ther* , 192, 327 (1975).
9 Schmitt, H., Schmitt, H., and Fénard, S., *Neuropharmacology*, 13, 347 (1974)
10 Page, I. H , Corcoran, A. C., Dustan, H. P., and Kopanyi, K , *Circulation*, 11, 188 (1955)

reviews

Is research management really as boring as this book makes it sound? "Finally he should desirably exercise some flair which stamps his department with an identity, making it recognisable as a unit with a reputation for excellence", writes Mr White at the end of less than a page devoted to the attributes of a research leader. Flair is the ingredient conspicuously missing from what is really a rather expensive handbook of information for the research manager, long on lists of research laboratories but very short on convincing examples of either successful or mismanaged research.

Never has there been a greater need than now for a book on the effective management of an inherently labour-intensive activity. Never has there been greater need for advice on how to make maximum use of scientific talent, by example and inspiration, and by providing scientists with efficient tools and support. Yet I looked in vain for specific examples of people who have succeeded in making better use of a scientist's time, or examples of balanced (and unbalanced) research portfolios. Where, at a time when those responsible for granting or rejecting applications for research funds are so well aware that funds are flowing not to the most deserving areas of research but to scientists who can write a convincing proposal, is advice on how to put forward your case for research? What are the differences between a good and a bad research proposal? To be more chari-

Competent if not inspired

David Fishlock

Effective Management of Research and Development. By P. A. F. White. Pp. x + 295. (Macmillan: London and Basingstoke, May 1975.) £10.00.

table, the author's most recent experience of research management, as a divisional head at the atomic weapons factory at Aldermaston, one of the most secret enclaves of science in Britain, has probably had an inhibiting effect on his readiness to supply or seek out the kind of details and examples that could have enlivened his book so much. But it scarcely excuses him from the charge of publishing out-of-date information. The long lists of research laboratories in Britain, given presumably to help the research manager with a problem to select a contractor who might help, contain names that no longer exist, and record budgets of four or five years ago, even for a public corporation such as the National Research and Development Council whose annual reports are readily available. One table offering the ratio of research funds spent in-house to those subcontracted, for 11 industries, is dated 1966-67.

When, however, the author drops the pretence of providing a handbook of up-to-date research data and concentrates on writing a textbook of research management, he stands on firmer ground. The latter half of his book starts with a chapter on the choice of a research and development portfolio—"probably the most important task of research management"—in which he sets out the principles, basically three, and reviews the sophisticated techniques which nowadays can help the manager to calculate economic benefit. Here I would cavil only at the brevity of the section on the stopping of research projects, acknowledged to be "one of the most difficult of a director's activities" yet commanding less than four pages when a whole chapter would have been fully justified. But it leads into further chapters on the control of projects, on efficiency and productivity in research and development, and on choosing research staff.

At the end of each chapter the author provides a brief but lucid summary of the points he has been trying to make. Rereading this review I think that the point I really want to make is that Mr White has almost totally neglected the human factor in an activity where, perhaps more than in any other field of endeavour, the difference in terms of success between competent and inspired research management—as between competent and inspired research—is a very big factor indeed. □

SINCE the previous volume in this series of symposia devoted to virology was published in 1968 progress in the field has been considerable. This compilation of 14 articles on the theme of control processes in virus multiplication is, therefore, an invaluable source of information for students and would-be students of animal virology.

Like other symposia in the series this is a collection of specially written review articles, rather than research papers, aimed at a readership of general microbiologists. This symposium deals principally with animal viruses. Only 4 of the 14 articles are concerned specifically with bacteriophages, and none of them considers the multiplication of plant viruses though there is a mention of certain structural aspects of these in an article by Showe and Kellenberger. The editors have to a large extent succeeded in keeping the articles within

the stated theme. Reviews which are not so confined include the first three which are intended to provide back-

Latest virology

Control Processes in Virus Multiplication. (25th Symposium of the Society for General Microbiology, London, April 1975.) Edited by D. C. Burke and W. C. Russell. Pp. viii + 449. (Cambridge University Press: London, New York and Melbourne, 1975.) £9.00; $27.50.

ground information to the subsequent reviews. Indeed, Dressler in a very readable article admits that very little is known about the control of DNA replication but explains the advantages available to DNA molecules which adopt a circular form for replication, and goes on to describe the way the

T7 bacteriophage genome replicates without adopting a circular form.

Some of the articles are not easily assimilated perhaps because they each deal with a different aspect (or closely related aspects) of control for all the main classes of animal virus; they may have been easier to assimilate if each had dealt with a different class of viruses but had explored all aspects of control known within that class.

I strongly recommend this comprehensive collection of authoritative review articles on a theme of great significance in the field of virology, and will conclude with a quotation from the editors' preface: " . . . it has been our intention to describe the 'state of the art' at the time of writing, and if the reader is more impressed on occasions by our lack of knowledge, then we trust he or she may be stimulated to repair the deficiency!" **A. C. R. Samson**

Tropical landforms

Tropical Geomorphology: A Study of Weathering and Landform Development in Warm Climates. (Focal Problems in Geography Series.) By Michael F. Thomas. Pp. xii+332. (Macmillan: London and Basingstoke; distributed in USA and Canada by Halsted Press.) £2.95.

THE terms bornhardt and inselberg reflect the keen interest taken by German scientists in tropical landforms during the late 19th century. The British came in strongly in 1911 with J. D. Falconer's account of the geology of northern Nigeria. Comprehensive summaries appeared later, the chief being S. Passarge's *Panoramen afrikanischer: Inselberg-landschaften* (1928) and Karl Sapper's remarkable *Geomorphologie der feuchten Tropen* (1935), and recently the French have produced much literature on tropical landscapes, including Jean Tricart's *Le modelé des régions chaudes, forêts ét savanes* (1965) which was translated into English by C. J. K. de Jonge in 1972.

Now Dr Thomas, having published numerous percipient articles, has produced the first full treatment of tropical geomorphology written originally in English. He balances the results of his own wide-ranging field studies against the findings of other warm-climate geomorphologists, and summarises carefully the issues that are fundamental to an understanding of the development of tropical landforms and regoliths. In addition, by analysing in detail weathering processes and their relationships to common morphometry, he ensures that the reader understands geomorphogenesis anywhere under warm bioclimatic conditions in which the work of wind, frost and waves is absent.

Part 1 contains five chapters on weathering and in these the author stresses the increased tropical rate of endothermic chemical reactions, the stability of vast horizontal surfaces, and the frequent occurrence of silicate minerals in rock exposures. The description of the processes are set out clearly and concisely, particularly the eluviation of silica and the formation of duricrusts and of deep-seated profiles.

Part 2 discusses the character and development of the tropical terrain and includes chapters on distinctive individual landforms, on hill-slopes, and on pediments and pediplanation. The text ends with a bibliography and three indexes.

Throughout, the author recognises that the processes undergo complex variations in intensity and that inherited or relict forms may be part of the general picture. The landforms characteristic of the tropics are shown to experience a continuity of sub-aerial morphogenesis over a prolonged period during which interruptions by tectonic and climatic pulses are localised in space and time. Dr Thomas makes the reader aware of the many problems ahead and of the great progress made during the last few decades. His concise analysis combines clarity of exposition with clear-cut diagrams and ample quantification so successfully that it deserves to become straightaway the standard text on the geomorphology of the humid tropics.

Robert P. Beckinsale

Thereby hangs a tail: a highly placed leopard watches for prey. From *Tigerland.* By Kailash Sankhal. (Collins: London, May 1975.) £3.50.

Antibiotic agents

Mechanism of Action of Antimicrobial and Antitumor Agents (Antibiotics, vol. 3.) Edited by J. W. Corcoran and F E. Hahn. Pp. xii+742. (Springer: Berlin and New York, 1975.) DM.188; $77.10.

THIS volume is the third of a series and provides up to date information on many of the classes of antibiotics described in the first volume published eight years ago. In spite of the inclusion of 46 chapters, it is not a complete review and classes of antibiotic such as the actinomycins and nitrofurans, which are missing, will form the basis for a future volume. Among the 3,500 or so references are to be found a sprinkling of 1973 quotations, so the book is reasonably up to date, which is a good achievement for a publication of this complexity.

The book deals with antibiotics defined in the broadest sense of the word and chapters are included on alkaloids and synthetic chemicals which are clearly like antibiotics in their mechanism of action. Descriptions are given of a great variety of agents which have properties ranging from antibacterial and antiparasitic, to antitubercular and antitumour, and agents which are primarily useful as laboratory probes for investigating biological processes are also discussed. Indeed, some chapters contain accounts of chemicals which seem to have no practical value and which, presumably, are still looking for some disease to cure.

A clear sign of the emerging interest in antibiotics as antitumour agents is evident from the first section of the book on agents that interfere with nucleic acid biosynthesis. The majority of the compounds described in this section have some antitumour properties, and though only bleomycin and members of the anthracycline group are of proven clinical value, it is apparent that this is an area in which new discoveries may well be made. A second section deals with agents that interfere with protein biosynthesis and a miscellaneous final section is devoted to agents that interfere with specific enzymes and with membrane or cell wall biosynthesis and with agents whose mechanism of action is unknown.

The editors have obviously given a lot of thought to the design of the book and to the selection of authors. What emerges is a pleasingly uniform series of chapters dealing with the mechanism of action of various antibiotics. With few exceptions, the chapters are authoritative and provide critical accounts rather than just a catalogue of reported work.

Mechanisms of selectivity are discussed in a few of the chapters. Ethidium, for example, is an antiparasitic agent probably because it interacts specifically with closed circular molecules of extrachromosomal DNA. The characteristic sensitivity of squamous cell carcinoma to bleomycin is explained by the fact that this tumour type concentrates the drug and is also unable to convert it to its non-toxic form. Unfortunately, many authors, having described the probable mechanism of action of a compound, make no effort to explain the basis of its selectivity. Why, for instance, should one intercalating agent be an antimalarial, another antitumour, and yet another just a poison? Perhaps the reasons for selectivity are still little understood, but a few comments by each author, even if only of a speculative nature, would have rounded off a good book.

All in all this is a fine volume which, combined with the promised volume dealing with agents not covered in this book, provides a comprehensive and critical survey of our present day knowledge of antibiotics and related compounds.

T. A. Connors

Natural and Synthetic Polymers: An Introduction. By Henry I. Bolker. Pp. xiv +688. (Dekker: New York, November 1974.) $29.75.

THE great increase in the number and type of synthetic polymers during the 25 years since the publication of K. H. Meyer's book under almost the same title as this one, and the great deal more that we now know about both synthetic and natural polymers, would make it a daunting task for anyone to follow precisely in Meyer's footsteps. Dr Bolker does not attempt to do so. Instead, he maintains wide coverage by making only brief statements about many of the polymers with which he deals, each of which could often be expanded into a whole book in itself; though, with his commendable conciseness, he manages to pack a surprisingly large amount of information into a small space.

His account differs from others I have read in basing the sequence in which the polymers are considered, not upon their chemistry or function, but upon the molecular geometry of their structure, passing from linear through branched to cross-linked architectures and, therefore, from simple to complex. This gives some natural polymers somewhat awkward neighbours but it has a logic about it which is appealing, which leads students gently by the hand (and this is why the book was written), and has a great advantage in bringing together in a unified way matter normally taught separately by 'natural product' chemists, by polymer chemists and by biologists. The upshot is a very readable book.

Linear polymers are dealt with in four chapters of which the longest (and the longest in the book) is the first, dealing with cellulose as the type linear homopolymer upon which polymer chemistry was for so long based. The discussion of branched polymers, also covered in four chapters, includes considerations of heteropolysaccharides and nucleic acids as well as the multitudinous synthetic copolymers. Cross-linked polymers include proteins (and the genetic code) and lignin. For each group of polymers, attention is paid to structure, to properties, to synthesis or biosynthesis, and to the use by man or the function in nature. Each chapter carries its own reference list and many of the great names of polymer science will be found here though the almost completely chemical approach has meant that some of the non-chemists are, sadly, not mentioned.

In the sections covering those aspects about which I know best the book is not up-to-date—the explosion of knowledge of polysaccharide structure during recent years, for instance, is hardly touched upon—but it would be surprising if every part of a book with such wide coverage could be 'stop press'. I personally would have been happier to have had more diagrams of the ball-and-spoke type rather than chemical formulae; they give a better appreciation of the three-dimensional geometry. And some of the diagrams could have been drawn more tidily. These are, however, minor criticisms of a book which skilfully takes students through a complex series of discoveries, which turns a maze into a garden path. It is to be recommended strongly to students not only of polymer chemistry but of physics and biology, and indeed mature scientists in these disciplines might profit from reading it. I have certainly profited myself. **R. D. Preston**

Polymers

Ionic Polymers. (Materials Science Series.) Pp. xii+416. Edited by L. Holliday. (Applied Science : London, 1975.) £14.00.

AFTER reading this book I not only share the editor's view that materials like the inorganic borates, phosphates and silicates should be regarded as polymers of a particular (ionic) type, I feel that the very existence of the book will make it impossible for authors of future texts on polymer chemistry to resist the inclusion of these inorganic compounds in what is clearly their proper context. The text, taken in order, gradually passes from comparatively orthodox—in polymer science terms—discussion of the synthesis and properties of certain predominantly organic macromolecules to descriptions of the sheet-like and three-dimensional structures of the inorganic polymers. I am left with more than a sneaking suspicion that if history had permitted organic polymer chemistry to precede the study of the silicates, the intentional preparation of the latter would have been hailed as a substantial achievement in polymer synthesis.

The eight chapters, taken together, give a pretty comprehensive view of ionomers, from detailed structural studies of the group to their commercial utilisation. The first chapter provides a general discussion, dealing with nomenclature and general properties. After that the text covers ionomers of the thermoplastic and elastomeric types and moves on through a range of materials of increasing ionic content to the longer standing polyelectrolytes and inorganic silicates, and so on. In the middle range, cements and soil-conditioning materials are found together with the metal dicarboxylates, for which the name 'halatopolymers' is coined—are they salts or polymers, or both?

Naturally, the various articles vary in approach but the important thing is the book as a whole : it not only fills a void in the literature of polymers, it also teaches a valuable lesson which should leave its mark on all future contributions. **A. D. Jenkins**

Molecular Behaviour and the Development of Polymeric Materials. Edited by A. Ledwith and A. M. North. Pp. 553. (Chapman and Hall : London, February 1975. Distributed in the USA by Halsted Press.) £12.00.

AT the research level a large proportion of new books consist of collections of review chapters, contributed by different authors. Where such collections are aimed at the specialist in a particular discipline, they are easily judged by the standards of that specialist. The present volume is more difficult to judge, since it has a different motivation, being compiled as a richly deserved tribute to Professor C. E. H. Bawn, on his retirement from the University of Liverpool. The review articles which form the book have been written by friends and former colleagues of Professor Bawn and their only other unifying theme is that they all lie within the boundaries of polymer science. The result is a somewhat heterogeneous book with topics that cover a very wide range of interests.

The 14 chapters comprise three reviews of polymerisation mechanisms, four broad reviews of specific polymer types (polybutadienes, other elastomers, polyolefins and polyurethanes), two reviews on the chemical reactions of polymers and five reviews on the structure and physical properties of polymers. All of the chapters have been written to a high standard and editorial monitoring has been good. I found the chapter by Small, on polyolefins, and the chapter by Ledwith and Sherrington, on polymers as catalysts, especially interesting, the former for its critical discussion of why so few polyolefins are commercially successful and the latter for its treatment of an underdeveloped area of great potential.

This is a book which is unlikely to be read by the specialist looking for new insight into his own discipline. Certainly in those areas where I can claim expertise, there is little which has not already appeared elsewhere. But for those wishing to get a feel for what is going on in a selection of other areas of polymer science this volume will provide much of interest. At the price (which is high but not excessive) few individuals will find enough of sufficiently urgent interest to wish to buy a personal copy but the book can be highly recommended as a library purchase. **N. C. Billingham**

Understanding of the developing child

Learning and the Development of Cognition. By Barbel Inhelder, Hermine Sinclair and Magali Bovet. Translated by Susan Wedgwood. Pp. xiv + 308. (Routledge and Kegan Paul: London, February 1975.) £4.95.

PIAGET'S theory is frequently presented as a rigid maturational system in which stage follows stage, more or less regardless of the child's social milieu, so that specific training procedures to speed up intellectual development are ineffective and pointless: either the child has the developmental structures to assimilate the material, in which case nothing substantially new can be acquired, or else those structures are not yet developed and no useful learning can take place.

No wonder that psychologists and educators alike, in their efforts to understand and to instruct the developing child, recoil from this altogether pessimistic picture and put it aside as irrelevant. No wonder, either, that any experimental evidence of successful intervention programmes in teaching mental operation at a younger age than commonly expected is flaunted as proving Piaget's theory wrong. Fortunately, the interpretation I have mentioned is but a caricature of the theory, born out of the unquestioned assumption that between a hereditarily given, unchanging potential and learning in the form of storing and coding environmental information there are no other factors contributing to the growth of intelligence. Piaget rejects both extremes of the heredity–environment controversy without lapsing into an interactionist stance in which one is locked in interminable arguments about the correct statistical proportions, but by proposing mental development as a psychological process altogether different from the learning of information or the unfolding of instinctual patterns.

This book, if nothing else, is a reminder that Piaget's theory is not at all aversive to a study of environmental input as it affects children's efforts at understanding their world. On the contrary, the investigations reported are predicated on the idea that developmentally suitable encounters will bring about intellectual progression; yet there is no concomitant desire of speeding up, rather of observing the developmental process in a concrete setting. The authors, who are Piaget's closest collaborators of many years, conceptualise this progression as a construction on the part of the child in the face of

fluctuations and conflicts between apparently divergent schemes of thinking. The encounters are devised precisely to expose children, if possible, to an awareness of this conflict and thus to bring about a restructuring of these schemes; in other words, a progression from a base of less or conflicting understanding to a stage at which the conflicting views are subsumed as nondisturbing facets of a more adequate understanding. As an illustration, in the comparison of a W-shaped line and a straight line, the length of the lines and the number and configuration of segmental units making up the lines can create a conflict in a child who does not fully coordinate the concept of length in relation to segmentation and unit element.

In all experiments the children were carefully selected as being in a transitional phase and tested before and after training; each child's performance was individually analysed and tentative categories of progress—or lack of it—were proposed. Though the methods of experimentation include the use of such familiar concepts as assessment of the quantity of a liquid, numbers, inclusion, conservation of substance and weight, the thinking activities to which the children were exposed are ingenious and original, not only in helping the child's progress, but also in helping us to understand and observe the process of development. In the foreword Piaget singles out some of the more important theoretical insights to be gleaned from this research which is frankly acknowledged to be but an exploratory beginning in the search towards the unravelling of the process of development.

Thus the book is another milestone in Genevan research and contains the clearest exposition so far of Piaget's elusive concept of developmental equilibration. One may add to this that the translation was supervised by one of the authors and is not full of mistranslations as are, unfortunately, many other works on Piaget's theory. But make no mistake, like other Genevan writings it is not easy reading and it does have weaknesses for which the Genevans can be so easily criticised: points of empirical and methodological inadequacy, incomplete protocols, theoretical overinterpretations. A reader who cannot see beyond these will no doubt be dissatisfied. But those who want to understand better the nature of environmental occasions leading to intellectual development, and who search for guidelines in worthwhile psychological research will find here an exciting theoretical framework, all the more so on account of its obvious practical and educational applications.

H. Furth

obituary

Joseph Hewitt, designer of the Hewitt satellite camera, died on May 21 at the age of 63.

Born at Leicester in 1912, Hewitt took a first degree in Botany at University College, Leicester in 1933, but after some research on the colorimetry of cells, he became convinced that optics was his métier, and he took a postgraduate course on technical optics at Imperial College, London in 1939. In 1942 he joined what is now the Royal Radar Establishment at Malvern, where he remained for the rest of his career, becoming leader of the photographic unit and also working on lasers. Hewitt will be particularly remembered as the designer of the Hewitt camera, a Schmidt-type instrument of 600 mm aperture with a 900 mm mirror and a field-flattening lens. Two of these cameras were made and are at present owned by the Ordnance Survey, for whom Hewitt acted as consultant for the past three years, after retiring from RRE in 1972. The cameras have proved to be the most accurate in the world for observation of artificial satellites, and the observations have been extensively used in geodetic and geophysical researches.

Sir William Hodge, FRS, ScD, FRSE, who was Lowndean Professor of Astronomy and Geometry and Master of Pembroke College, Cambridge, from 1958–70, has died at the age of 72.

Born at Edinburgh and educated at St John's, Cambridge, Sir William spent most of his working life in Cambridge, with the exception of five years as a lecturer at Bristol and a year at Princeton. After holding a research fellowship at St John's he became a staff fellow of Pembroke, until in 1936 he became Lowndean Professor. In 1958 he was elected Master of Pembroke, and, together with Lady Hodge, did much to preserve a friendly atmosphere in the college. Hodge's main mathematical contributions were in the field of algebraic geometry. His theory of harmonic integrals has had a profound influence on the development of geometry in the past 30 years. After the war he played a leading role in the formation of the International Mathematical Union, serving as vice-president from 1954–58. He was one of the instigators of the British Mathematical Colloquium, and was also president of the London Mathematic Society, the Mathematical Association and the Cambridge Philosophical Society. He was elected a fellow of the Royal Society at the early age of 35, and was physical secretary from 1957–65 and a vice-president from 1959–65. Recognition for his public services came in 1959 when he was knighted. The Royal Society awarded him its Royal Medal in 1957 and the London Mathematical Society gave him the de Morgan Medal in 1959.

announcements

Awards

The 1975 **Glaxo Travelling Fellowships** for British science writers have been awarded to **Dr** and **Mrs Oliver Gillie** (joint award), **Michael Barnes** and **Frank Walsh.**

Appointments

W. Ashworth has been appointed pro-vice-chancellor of the University of Bristol.

W. F. Wood has been appointed dean of the faculty of environmental studies at Heriot-Watt University.

Miscellaneous

Vision research. Further information about the research fellowships being offered by the Worshipful Company of Spectacle Makers, may be obtained from: The Clerk of the Company, Mr C. J. Eldridge, Apothecaries' Hall, Blackfriars Lane, London EC4V 6EL, UK.

International meetings

September 21–25, **Mammalian cell culture and its application,** Birmingham, Alabama (Professor R. T. Action, Symposium Chairman, Department of Microbiology, University of Alabama in Birmingham, University Station, Birmingham, Alabama 35294).

September 21–26, **Electrochemistry in non-aqueous solutions,** Baden, Austria (26th ISE Organising Committee, Verein Osterreichischer Chemiker, Eschenbachgasse 9, A1010 Vienna, Austria).

September 21–27, **Space and energy,** Lisbon, Portugal (International Astronautical Federation, 250 Rue Saint-Jacques, 75005 Paris, France).

September 22–24, **Engineering in the ocean environment,** San Diego (Institute of Electrical and Electronics Engineers, Technical Activities Board, 345 East 47th Street, New York, New York 10017).

September 22–24, **Aerospace simulation facilities,** Ottawa (E. S. Hanff, National Research Council of Canada, Montreal Road, Ottawa, Canada K1A 0R6, Canada).

September 22–24, **Turbulence in liquids,** Missouri (Professor G. K. Patterson, Chemical Engineering Department, University of Missouri-Rolla, Rolla, Missouri 65401).

September 22–25, **Origins and evolution of language and speech,** New York (Conference Department, The New York Academy of Sciences, 2 East 63rd Street, New York, New York 10021).

September 22–26, **Microdosimetry,** Verbania Pallanza, Italy (Secretariat of the 5th Symposium on Microdosimetry, Commission of the European Communities DG XII, Biology Division, Dr H. G. Ebert, 2QO rue de la Loi, B1040 Brussels, Belgium).

September 22–26, **Ferroelectricity,** Zurich (Secretariat EMF3, Zurich

Laboratory of Solid State Physics, Swiss Federal Institute of Technology, Honggerberg CH8049, Zurich, Switzerland).

September 22–26, **Vertebrate palaeontology and comparative anatomy,** Edinburgh (S. M. Andrews, Royal Scottish Museum, Chambers Street, Edinburgh EH1 1JF, UK).

September 23–25, **Cybernetics and society,** San Francisco (L. S. Coles, Artificial Intelligence Center, Mento Park, California 94025).

September 23–25, **Instrumentation in oceanography,** Bangor, Wales (Conference Department, Institution of Electronic and Radio Engineers, 8–9 Bedford Square, London WC1 3RG, UK).

September 24–25, **Regulation of biosynthesis of bacterial walls and membranes,** Newcastle upon Tyne (The Meetings Assistant, Society for General Microbiology, Harvest House, 62 London Road, Reading, Berkshire RG1 5AS, UK).

September 24–26, **Polymer physics,** Shrivenham, UK (Mrs H. Higdon, Physics Department, Brunel University, Uxbridge, Middlesex UB8 3PH, UK).

September 25, **Electron spin resonance bioenergetics,** London (Dr J. Barber, Botany Department, Imperial College, London SW7, UK).

September 29, **Chromatin,** Glasgow (Dr A. J. MacGillivary, Beatson Institute for Cancer Research, 132 Hill Street, Glasgow G3 6UD, UK).

September 30–October 6, **Biometeorology,** Israel (Mr Y. Lumas, Department of Agricultural Meteorology, Meteorological Service, PO Box 25, Bet-Dagan, Israel).

Reports and publications

Great Britain

Annual Report of the Grassland Research Institute for 1974. Pp. viii+163. (Hurley, Maidenhead, Berkshire: The Grassland Research Institute, 1975.) £2. [26
Report of the Tropical Products Institute, 1972/1974. Pp. v + 96 + 12 plates. (London: Tropical Products Institute, Publications Section, 127 Clerkenwell Road, EC1, 1975.) 90p net. [26
Medical Research Council. Criteria for Controlling Radiation doses to the Public After Accidental Escape of Radioactive Material. Pp. vii + 48. (London: HMSO, 1975.) £1.30 net. [26
Home-Grown Cereals Authority. Progress Reports on Research and Development, 1973/1974. Pp. 23. (London: Home-Grown Cereals Authority, Hamlyn House, Highgate Hill, N19, 1975.) [26
The Work of the Rutherford Laboratory 1974. Edited by J. R. Smith and F. M. Telling. (Science Research Council.) Pp. 207. (Chilton, Didcot: Science Research Council, Rutherford Laboratory, 1975.) [46
Avon Council on Alcoholism. Fifth Annual Report 1975. Pp. 31. (Bristol: Avon Council on Alcoholism, 1975.) 40p. [56
The British Industrial Biological Research Association. Annual Report for 1974. Pp. 56. (Carshalton, Surrey: The British Industrial Biological Research Association, Woodmansterne Road, 1975.) [56

British Caenozoic Fossils (Tertiary and Quaternary). Fifth edition. Pp. vi + 132 (44 plates). (London: British Museum (Natural History), 1975.) 50p. [56
University of Oxford. Ashmolean Museum—Report of the Visitors, 1973/1974. Pp. 63. (Supplement No. 3 to the *University Gazette,* Vol. cv, April 1975.) (Oxford The University, 1975.) £1.50. [66
Families Without Hope: A Controlled Study of 33 Problem Families. By W. L. Tonage, D. S. James and Susan M. Hillam. (*British Journal of Psychiatry* Special Publication No. 11.) Pp. xi + 156. (Ashford, Kent: Headley Brothers, Ltd., 1975. Published by authority of the Royal College of Physicians.) £4; $12. [96
Soil Survey. Soil Survey Field Handbook: Describing and Sampling Soil Profiles. Compiled and edited by J. M. Hodgson. (Technical Monograph No. 5.) Pp. vi + 97. £1.40. Soil Survey Laboratory Methods. Edited by B. W. Avery and C. L. Bascomb. (Technical Monograph No. 6). Pp. vi + 83. 60p. (Harpenden, Herts: Soil Survey, Rothamsted Experimental Station, 1974.) [96
Imperial College. Department of Metallurgy and Materials Science—Research in Progress 1974/1975. Pp. 24. (London: Imperial College, Department of Metallurgy and Materials Science, 1975.) [96
Forestry Commission. Leaflet No. 58: The Large Pine Weevil and Black Pine Beetles. By T. M. Scott and C. J. King. Pp. 12. 23p. Leaflet No. 59: Hydrostatic Skidder. By W. O. Wittering. Pp. 10. 27p. Leaflet No. 60: Selection of Conifer Seed for British Forestry. By J. N. Kennedy. Pp. 6. 8p. Forest Record No. 93: Cross Country Vehicles in Forestry. By D. H. Wallace. Pp. 28. 40p. Forest Record No. 94: Biology of Dutch Elm Disease. By J. N. Gibbs. Pp. 9. 23p. Forest Record No. 95: Wood Resources and Demands—a Statistical Review. By A. J. Grayson. Pp. 14. 25p. Forest Record No. 96: Beech Bark Disease. By E. J. Parker. Pp. 15. 25p. Forest Record No. 97: Forest Site Yield Guide to Upland Brtain. By R. J. N. Busby. Pp. 13. 40p. Forest Record No. 100: Dutch Elm Disease Survey, 1972/1973. By J. N. Gibbs and R. S. Howell. Pp. 26. 30p. Bulletin No. 50: Fifty Years of Forestry Research. By R. F. Wood. Pp. viii + 134. £1.75 net. (London: HMSO, 1974.) [106

Other countries

Yale University: School of Forestry and Environmental Studies. Bulletin No. 85: Toward the Future Forest—Applying Physiology and Genetics to the Domestication of Trees. By J. H. Rediske, F. Thomas Ledig, Peter S. Carlson, Thomas B. Rice and Richard P. Pharis. Edited by F. Thomas Ledig. Pp. 80. (New Haven Conn.: Yale University, 1974.) $3. [26
World Health Organization. Technical Report Series. No. 558: Community Health Nursing—Report of a WHO Expert Committee. Pp. 28. Sw.fr.4. No. 560: Chemical and Biochemical Methodology for the Assessment of Hazards of Pesticides for Man—Report of a WHO Scientific Group. Pp. 26. Sw.fr.4. No. 565: WHO Expert Committee on Biological Standardization—Twenty-sixth Report. Pp. 72. Sw.fr.7. (Geneva: WHO; London: HMSO, 1974 and 1975.) [26
Organization for Economic Co-operation and Development, Paris. OECD Informatics Studies No. 8: Applications of Computer/Telecommunications Systems. (Proceeding of the OECD Seminar, November 13–15, 1972.) Pp. 271. (Paris: OECD; London: HMSO, 1975.) F36; £3.60; $9. [26
Battelle Memorial Institute. The President's Report and Annual Review, 1974. Pp. 48. (Columbus, Ohio: Battelle Memorial Institute, 505 King Avenue, 1975.) [26
World Health Organization. Cost and Benefit of Fluoride in the Prevention of Dental Caries. By G. N. Davies. (WHO Offset Publication No. 9.) Pp. 91. Sw.fr.20. Manual on Personal and Community Protection Against Malaria in Development Areas and New Settlements. Prepared by the WHO Division of Malaria and Other Parasitic Diseases. (WHO Offset Publication No. 10.) Pp. 49. Sw.fr.14. (Geneva: WHO; London: HMSO, 1974.) [36
Australia: Commonwealth Scientific and Industrial Research Organization. Annual Report of the Marine Biochemistry Unit 1973/1974, July 1—June 30. Pp. 27. (Sydney: CSIRO, 1975.) [46
Environment Canada. Fisheries and Marine Service Technical Report No. 638: Effects of Several Experimental Diets on the Growth of Rainbow Trout in Salt Water. By A. M. Sutterlin, H. Barchard and G. Melvin. Pp. 8. (St. Andrews, New Brunswick: Research and Development Directorate. Biological Station, 1975.) [46
Smithsonian Contributions to Zoology, No. 187: Communication in Red Fox Dyads—a Computer Simulation Study. By Gerald Gene Montgomery. Pp. iii + 30. (Washington, DC: Smithsonian Institution Press, 1974. For sale by US Government Printing Office.) 95 cents. [46
Australia: Commonwealth Scientific and Industrial Research Organization. Serial Publications. Monographs and Pamphlets 1974. Pp. 124. (East Melbourne: CSIRO, Central Information, Library and Editorial Section, 1975.) [46
The Australian Mineral Development Laboratories. Fourteenth Annual Report, 1974. Pp. 40. (Frewville, South Australia: The Australian Mineral Development Laboratories, 1975.) [56
US. Department of Health Education and Welfare —National Institutes of Health. National Cancer Program. Report of the National Cancer Advisory Board. Pp. 13. Report of the Director. Pp. 92. Report of the President's Cancer Panel. Pp. 12. Appendices to the National Cancer Program Operational Plan—Individual Program Descriptions. Operational Plan. FY1976–1980. (Bethesda, Md. US Department of Health, Education and Welfare. Public Health Service. National Institutes of Health, 1974.) [66

Canada: Department of Energy, Mines and Resources. Geological Survey of Canada. Paper 74–13: Carcajou Canyon Map-Area, District of Mackenzie, Northwest Territories. By J. D. Aitken and D. G. Cook. Pp. vi + 28. $3. Paper 74–28: Ordovician Strates of the Hudson Bay Lowlands. By L. M. Cumming. Pp. 93. $4.20. Paper 74–41: Studies in "Standard Samples" of Silicate Rocks and Minerals. Part 4: 1974 Edition of "Usable" Values. By Sydney Abbey. Pp. 23. $2. Paper 75–6: The Geosciences in Canada—1974, a Status Report prepared by the Canadian Geoscience Council. Edited by E. R. W. Neale, A. C. Clzgue and H. R. Wynne-Edwards. Pp. 51. $2.40. Paper 75–18: Under-Ice Scuba Techniques for Marine Geological Studies. By Patrick McLaren and David Frobel. Pp. 13. $3.60. Paper 75–20: Manifile—The University of Manitoba Computer-Based File of World's Nonferrous Metallic Deposits. By P. Laznicka. Pp. 11. $2. Paper 75–22: Development of a Computer-Based File on Oil and Gas Pools. By G. J. Dickie and G. D. Williams. Pp. 14. $2. (Ottawa: Information Canada, 1974 and 1975.) [66
Institut National de la Recherche Agronomique. Service d'Etude des Sols et de la Carte Pedologique de France. Carte Pedologique de France au 1/100,000. Toulon—P23. Notice Explicative. Par J. Portier. Pp. 125. (Versailles: Institut National de la Recherche Agronomique, 1974.) 53.50 francs. [96
The Nucleus: Annual Review of the Science Foundation for Physics and the School of Physics Within the University of Sydney. Pp. 24. (Sydney: The University, 1975.) [106
Crown-of-Thorns Starfish Seminar Proceedings, Brisbane, 6 September 1974. Pp. vi + 191. (Canberra, ACT: Australian Government Publishing Service, 1975.) [106
British Columbia Research Council. BC Research—Annual Report 1974. Pp. 24. (Vancouver: British Columbia Research Council, 1975.) [116
Republic of Botswana. Annual Report of the Geological Survey and Mines Department for the year 1972. Pp. 59. 50 cents. Annual Report of the Geological Survey and Mines Department for the year 1973. Pp. 54. 50 cents. Geological Survey Department Bulletin No. 4: Earth Resources Technology Satellite —1. Coverage of Botswana received at Geological Survey Department as at April 1974. Compiled by S. M. Hutton, Pp. 21. (Lobatse, Botswana: Geological Survey Department, 1974.) [116
Publications du Centre National pour l'Exploitation des Oceans (CNEXO). Serie: Rapports Scientifiques et Techniques. No. 19: Variabilite Saisonniere de la Couche Homogene dans le Golfe de Gascogne. Par Yves Desaubies. Pp. 33. No. 20: Étude Hydrologique et Variations Saisonnieres dans la Proche Atlantique en 1972. Par B. Fruchaud. Pp. 44. (Paris-Cedex: CNEXO, Boite Postale 107, 1975.) [116
Republic of South Africa. Annual Report of the Division of Sea Fisheries for 1972, No. 40. Pp. 29. (Sea Point, Cape Town: Sea Fisheries Branch, 1974.) [116
European Organization for Nuclear Research-CERN. CERN 75–4: A Comparison of the Radiation Damage of Electronic Components Irradiated in Different Radiation Fields. By K. P. Lambert, H. Schonbacher and M. Van de Voorde. Pp. 18. (Geneva: CERN, 1975.) [116
Acupuncture Anesthaesia in the People's Republic of China. By James Y. P. Chen. (A Publication of the Geographic Health Studies, John E. Fogarty International Center for Advanced Study in the Health Sciences. US Department of Health, Education and Welfare, Public Health Service, National Institutes of Health.) Pp. xi + 105. (Washington, DC: US Government Printing Office, 1975.) $1.80. [136
World Health Organization. Annual Report of the International Agency for Research on Cancer, 1974. Pp. 132. (Lyon: International Agency for Research on Cancer, WHO; London: HMSO, 1975.) Sw.fr.10. [136
Canada: Department of Energy, Mines and Resources. Geological Survey of Canada. Paper 74–2: Age Determinations and Geological Studies: K–Ar Isotopic Ages, Report 12. By R. K. Wanless, R. D. Stevens, G. R. Lachance and R. N. D. Delabio. Pp. 72. $3.50. Paper 75–24: Some Observations on the Solubility of Gold. By R. W. Boyle, W. M. Alexander and G. E. M. Aslin. Pp. 6. $2.40. (Ottawa: Information Canada, 1974 and 1975.) [136
Mitteilungen aus der Biologischen Bundesanstalt fur Land- und Forstwirtschaft, Berlin-Dahlem, Heft 163: Krankheiten und Schadlinge an Getreide, Olfrucht- und Futterpflanzen. Pp. 133. (Berlin-Dahlem: Biologischen Bundesanstalt fur Land- und Forstwirtschaft, 1975.) DM 14. [166
Guide to International Marine Environmental Data Services. Pp. 32. (Paris: Unesco, 1975.) [166
National Multiple Sclerosis Society. 1974 Annual Report. Pp. 32. (New York: National Multiple Sclerosis Society, 257 Park Avenue South, 1975.) [166
World Health Organization. Technical Report Series No. 567: WHO Expert Committee on Specifications for Pharmaceutical Preparations—Twenty-fifth Report. Pp. 115. (Geneva: WHO; London: HMSO, 1975.) Sw.fr.9. [176
Smithsonian Contributions to Zoology, No. 194: The False Spider Mites of Northwestern and North Central Mexico (Acarina: Tenuipalpidae). By Edward W. Baker, Donald M. Tuttle and Michael J. Abbatiello. Pp. iii + 23. (Washington, DC: Smithsonian Institution Press, 1975.) [176
World Health Organization. Technical Report Series, No. 561: Ecology and Control of Vectors in Public Health—Twenty-first Report of the WHO Expert Committee on Insecticides. Pp. 35. (Geneva: WHO; London: HMSO, 1975.) Sw.fr.6. [186
Australia: CSIRO. Annual Report of the Division of Textile Physics, 1973/1974. Pp. 31. (Sydney: CSIRO, 1975.) [206

August 7, 1975

Whitlam, Connor and Cameron

AUSTRALIAN scientists have been through a deeply traumatic period, as Peter Pockley reports (p. 448), and it is by no means clear that the somewhat ambiguous statement of Mr Whitlam on July 1 will be the end of the matter. The issues at stake deserve to be understood internationally, if only because they exemplify the sort of problems that are bound, sooner or later, to beset a country whose policy for the support of science has placed emphasis on unity by means of the large, some say monolithic, CSIRO (Commonwealth Scientific Industrial Research Organisation) with 6,600 staff spending £50 million annually, and a ministry trying to coordinate science.

CSIRO has traditionally enjoyed a high degree of autonomy in the running of its affairs. It reports direct to the Minister for Science, not through the ministry's secretariat, and thinks of itself as 'scientists run by scientists'. Promotion tends to be more by merit than by seniority. All of this has made CSIRO scientists wary of their status; to be moved into the public service sector and to report into the bureaucracy of a sectoral ministry would, they believe, seriously alter their style of doing things. It would also choke the inter-disciplinary links which have been carefully nurtured in the last few years.

By themselves such arguments might simply be seen as defences of a cosy *status quo* and if used in normal times would have to be offset against other considerations. But in this instance the removal of roughly 500 staff from the jurisdiction of the Minister for Science (Mr Cameron) to that of the Minister for Minerals and Energy (Mr Connor) has some more disturbing aspects.

First, the tasteless manner in which it was done gives no confidence that any type of consensus politics was being practised. To act unilaterally and hastily is, of course, Mr Whitlam's privilege as Prime Minister, but it is one which inevitably leaves a feeling that the multilateral discussion has had to be short-circuited because the logic doesn't stand up too well to examination.

Second, the ambitions of Mr Connor, although relatively little mentioned in the furore that followed the decision, are not those that endear him to many scientists. His low profile throughout the affair, his failure to speak out on his own reasons for wanting to annexe portions of CSIRO and his undoubted aspirations to run a very large and very Australian resources empire have left him with few supporters in the scientific community, even though his fascination with technology is unquestioned.

Third, and undoubtedly most important, there is much despair that the careful exercises during the past two years of Mr Bill Morrison (former Minister for Science) in attempting to enunciate a science policy for Australia could be so easily ignored. Only in January 1975, Mr Whitlam, on introducing the White Paper establishing the Australian Science and Technology Council (ASTEC), harangued scientists on the need to take science policy more seriously. And yet this decision was made without reference to ASTEC, in the absence of any indication in last year's OECD report on Australian science policy that CSIRO should be carved up, and without even the chairman of CSIRO being notified in advance. It will be difficult to get Australians to give up their time to thinking about the governing of science in future. And if the Department of Minerals and Energy is allowed to annexe what it wants, this could be a precedent for similar acquisitions by at least half a dozen other departments who have scientific needs close to CSIRO's interests.

In the midst of all this, Australia acquired a new Minister for Science and Consumer Affairs in Mr Clyde Cameron. There could only be sympathy for him at first at arriving to find a burglary had just taken place. But since then he has been travelling and his first major speech in the new job was delivered in New Delhi to the Indian National Science Academy. It gives grounds for concern. He declares " . . . science and technology is now responsible for activities that are polluting and poisoning the world's atmosphere, ionosphere and even stratosphere. . . . At the rate we are now moving, it will soon be possible to fix a timetable for the ultimate end of human life itself. . . . Australia wants its scientists to do two things: one to concentrate on those areas which will improve the quality of human life, not just in Australia, but everywhere. And secondly, to [concentrate on] research that will enable Australia to help remedy the incalculable damage which the abuse and misuse of scientific knowledge has already caused to nearly every country in the world. ASTEC will direct its attention to these two objectives." ASTEC, CSIRO and the academic community are in for a difficult time ahead.   ☐

international news

THE Australian Government and public, and not least the scientists themselves, have been amazed by the intensity and unanimity of the fight by CSIRO staff to retain the integrity of their organisation following the raid by the Minerals and Energy Department to take over the CSIRO's Minerals Research Laboratories and Solar Energy Energy Studies Unit. At the time of writing, a compromise appears to have been reached, albeit one open to alternative interpretations, and CSIRO has at least held its ground.

Among the many problems faced by the CSIRO Executive and supporters in the wider public in mounting their fight has been the simultaneous battle of Mr. Whitlam's government to survive in power. Politics in Australia has been turbulent in Labour's 2½ years in office, but the last few weeks have been remarkable for the severity of the battering taken by the government, mostly of its own making. No sooner had Mr Whitlam finalised his new ministry on June 6, by persuading Mr Clyde Cameron to accept the Science and Consumer Affairs portfolio (and, at the same time, announced amputation of CSIRO), than the byelection campaign began to go sour in the safe Labor seat of Bass in Tasmania vacated by Mr Lance Barnard whose resignation had precipitated the Cabinet reshuffle. Labor lost the seat in a landslide to the Liberals at a time when the CSIRO fightback was gathering great momentum.

The Prime Minister and his Minerals and Energy Minister, Mr Rex Connor, were hardly approachable on anything, let alone the CSIRO affair and the scientists' campaign was waged largely at arm's length against a silent adversary. Without a day's respite from their drubbing in Bass, the government was pushed into its greatest crisis when a long-simmering row about unorthodox ways of raising overseas loans of "petrodollars" (to the tune of $4,000 million) came to a head. The bulky Mr Connor (who does not delight in his popular nickname of "The Strangler") and the nice but naive Deputy Prime Minister, Dr Jim Cairns (demoted from the Treasury to Environment), were the central targets of attack from the Liberal Opposition and the media.

Finally, Mr Whitlam sacked Dr Cairns, a drastic enough move in itself, but this was insufficient to keep the

CSIRO holds ground in fightback

from Peter Pockley, Sydney
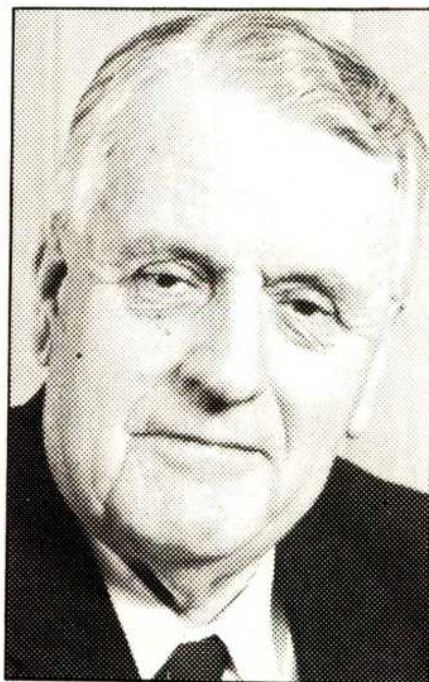
Connor : hardly approachable.

wolves at bay, and Parliament was recalled for a special session. One of the dozens of members of Parliament and Ministers who came scurrying back from overseas to support their sides in the crisis debate on July 9, was Mr Cameron who had been visiting Delhi, Moscow and Paris, far from the madding crowd of CSIRO staff. He had not been missed, though, for the Acting Minister for Science and Consumer Affairs, Dr Moss Cass, a quietly persuasive politician with a knowledgeable and sympathetic record on science policy matters, had taken up the scientists' case with drive and some effect.

Before Mr. Cameron arrived back in Australia, Mr Whitlam had issued a brief statement on July 1, which went some way to meeting the scientists' demands for autonomy in staffing conditions from the Australian Government Public Service. The Prime Minister said "that he wanted to make it clear that the fact that the Minister

for Minerals and Energy was responsible for those aspects of CSIRO's research work (minerals and solar energy) did not entail the transfer of the CSIRO's staff concerned to the Public Service. These staff would remain employed under the Science and Industry Research Act and it is not proposed that they should be transferred."

That this statement leaves much open to doubt is shown by the cautious statement issued the following day by the CSIRO Chairman, Dr Jerry Price, who has worked with great energy to defend his organisation. Dr Price said that CSIRO welcomed the announcement, and then went on to say: "The Executive of CSIRO is now examining the administrative machinery involved to put into practice the division of ministerial responsibility as outlined in the administrative arrangements order." For the first time CSIRO formally accepted that the administrative order "makes clear the movement of ministerial responsibility for matters relating to minerals and solar energy research from the Minister for Science and Consumer Affairs, Mr Clyde Cameron, to the Minister for Minerals and Energy, Mr R. F. X. Connor".

But, the puzzlement over how CSIRO can work to two Ministers remains. Mr Whitlam's announcement could be consistent either with CSIRO remaining intact as an organisation, or with a separate CSIRO-like organisation being formed, under the Minister for Minerals and Energy, from the transfer of the relevant CSIRO staff without change in their working conditions. The Minerals and Energy Department are understood to have worked on draft legislation to give effect to the latter alternative, but, to date, they have not showed their hand. Showing hands is not part of that Department's style, and in any case their energies have been consumed by the defence of their Minister and their equally redoubtable Departmental Head Sir Lenox Hewitt, who, apart from the tragic Dr Cairns, have been the central figures in the row over the petrodollars loans (which never were —such is the fate of unsuccessful borrowers).

Mr Connor could also obtain a stake in CSIRO's operations through nominating members of the Executive—the next full-time vacancy will arise in January 1976, but whether Mr

THIS account would be incomplete without a survey of the two years leading up to the raid on CSIRO. This story is only beginning to emerge as CSIRO collectively gains confidence to speak a little louder than before the crisis.

Mr Connor is one of the few Australian politicians to proclaim a personal interest in science. Modern technology is said to fascinate him deeply—his vision of a national pipeline grid, under public ownership, for the distribution of Australia's none-too-plentiful natural gas supplies has been one expression of his apparent belief that to apply technology on the grand scale is to control the development of society. Mr Connor and Sir Lenox Hewitt have shown single-minded dedication to their aim of coordinating, through government or legislative controls, all principal aspects of mining and energy policy, but their ways of achieving these ends have not been marked by their encouragement of public information and discussion. Perhaps they planned from the outset that gaining the control and exercising close direction of major research facilities in the minerals and energy fields were essential elements of their strategy—in many ways a sensible aim if considered in isolation—but they played their cards close to their chests.

In 1973 (the first year of Labor rule) mining appeared to be out of favour with the government. Overseas ownership and "rape of national resources" were the bogies. The CSIRO leadership, struggling hard to read the new government's mind and not finding itself closely in tune with the Science Minister Mr Morrison, decided to slow the rate of growth of their minerals research effort. This effort had flourished in the late 60s in the wake of Australia's mining boom; CSIRO had created new mining-oriented Divisions, such as Mineral Physics, and had grouped old and new Divisions together as the Minerals Research Laboratories under a Director, Mr Ivan Newnham.

Research into energy was partly located in these laboratories, for example, investigations into coal, but such areas of work were largely dispersed throughout the CSIRO organisation. However, a separate Solar Energy Unit was formed, under Mr Roger Morse, early in Labor's first term of office, a move popular with Mr Morrison and the environmental lobby. This was a modest operation, and Mr Connor was able, unbeknownst probably to CSIRO, to start building a case for a single minerals and energy research organisation, a case which he may have thought politically unbeatable because of the brakes put by CSIRO on minerals research (demonstrable from budget figures) and the organisation's supposed neglect of energy research (demonstrable from the CSIRO establishment chart and some inside information).

From early 1973 CSIRO began to make a series of formal approaches to the Minerals and Energy people seeking joint meetings between senior researchers to define areas of cooperation and rationalisation for the achievement of government objectives. The reception was cool—in the case of the original letter no acknowledgement was received for six months, in marked contrast to the response from other new Departments established under Labor with whom scientific cooperation was rapidly and substantially put into effect, e.g. with Environment, and Urban and Regional Development.

Within a year, CSIRO convened its own in-house conference on solar energy with papers from researchers both at the centre and periphery of problems of energy conversion and storage, ranging from photosynthesis to wind machines and low grade heating devices. Minerals and Energy gained the list of delegates and their topics, and later (after the June 5 announcement) used it as a check list on CSIRO's willingness to relinquish their staff in these areas.

Meanwhile, the OECD examiners arrived to analyse Australia's science and technology policies in March and April 1974. They were given extensive cooperation by the Ministers and Departments of Science, Health and En-vironment, but they let it be known that Minerals and Energy did not seem to welcome them, Mr Connor and Sir Lenox Hewitt are reported to have declined to see the OECD team who, in their August 1974 report, recorded that they were unable to identify any energy policy at the national level. This bald statement was well considered by the politically oriented OECD people, but it drew a vitriolic Parliamentary attack on their report from Mr Connor, which at least had the virtue of spelling out what the Minister believed to constitute a policy for energy.

By September, Mr Connor had begun to show his hand. He wrung a joint statement out of Science Minister Morrison to the effect that Minerals and Energy was "to become involved in the implementation of the results of solar energy research". While CSIRO would continue its research in low-grade heat conversion, Minerals and Energy "would take over the development phase of those CSIRO research results which were approaching practical realisation".

Around this time, the Minerals and Energy Department asked that CSIRO provide a list of staff working in the minerals and energy field (they already held what they believed was the solar energy part of this list). For this purpose, Sir Lenox Hewitt paid a visit to the Canberra headquarters of CSIRO; Dr Price, the Chairman, was absent and he was given no satisfaction by the two members of the Executive who received him.

By November 1974 some contact was restored but not at the level of the CSIRO Executive. Discussions, which the Executive later said they thought were of an informal, exploratory nature leading to possible liaison, were held between the Director of the Minerals Research Laboratories and Minerals and Energy. By this time, Professor Harry Messel, an Atomic Energy Commissioner, had become Mr Connor's adviser on nuclear and other scientific matters; he became involved in the build-up to the Prime Minister's announcement on June 5, which so stunned the CSIRO staff.

Whitlam's government can survive until then is now open to daily speculation. Even if the government survives, Mr Connor's power base may be eroded because of his part in the loans crisis.

When the next history of CSIRO is written, the researchers will have unaccustomed ease in obtaining documents for the 25 crucial days for CSIRO spanning the period June 5 to July 1, the dates of the Prime Minister's two statements. The official communication network and the un-official grapevine within the organisation worked overtime to keep the scattered staff up-to-date with latest developments.

The press came to realise that this aspect of science policy at least was big news—probably the first time in Australian history that science policy has rated front-page and editorial treatment. Australian scientists, notable for their caution about dealing with the press (there are no full-time science correspondents on Australian papers), began to brief journalists like long-lost allies. The crisis created a flood of general information about CSIRO which should never have been so cautiously damned up for so long. But, while the press were remarkably generous in their coverage of the CSIRO side of the story, it was notable that the reporters who covered it—necessarily only political and general journalists—were struggling to find in their memories or files any arguments about scientific achievements to support their reports.

In passing, it should be noted that

whatever the final outcome, CSIRO's approach to its publicity must now surely change to a more open, if not aggressive stance. Some (but by no means all) of CSIRO's present troubles with the government could have been averted by a more accurate reading of the political cimate over the past two-and-a-half years. On the other side, some of the government's embarrassment from a public confrontation with an essentially non-political body like CSIRO could have been avoided by a better understanding of the nature of scientific endeavour and researchers themselves. Facts about Australia's achievements and potential in research are not common currency, even among local scientists, let alone politicians and bureaucrats. Each side of the confrontation has lacked information and comprehension of the other. Despite its debilitating effects on the short-term, then, it is possible that the open fight over 25 days did a power of good in bringing political realities sharply home to the scientific community.

Once placed in the fire, CSIRO staff learned fast the tough business of political pressure. For instance, once they noticed that the government-financed Australian Broadcasting Commission, despite a swag of science programmers on its staff, had given scant attention to the CSIRO story at a time when the press were headlining it daily, direct pressure was placed on the ABC hierarchy. A special radio programme followed, with unanimous statements of support for keeping CSIRO intact from some unlikely but influential sources. As implied already, CSIRO is not immune to criticism, but not a whiff of this emerged in the public debate.

Reportedly, Mr Connor, Sir Lenox Hewitt and their adviser Professor Harry Messel (of Sydney University) had been assured during the planning of their raid on CSIRO that the Minerals Research and Solar Energy staff subject to transfer to the Minerals and Energy Department would welcome the charge. (It is difficult, at this stage, to be certain of the origin of this advice, but fingers have been pointed sharply in only one or two directions.) That such advice was catastrophically wrong, and that the CSIRO staff have a solid if quiet pride in their organisation, has been shown by the complete lack of statements of support for the Connor/Hewitt/Messel move from within or without CSIRO. Such was the anger generated among some CSIRO people that there were underground moves to turn the raid back on the raiders. It is remarkable that, if there were good arguments in favour of consolidating all minerals and energy research, none have emerged publicly, even from the Minister involved or,

indirectly, by the much-favoured technique of off-the-record leaks to the press.

From June 12 onwards, meetings in laboratories and groups of laboratories regularly drew hundreds of staff from across the board of CSIRO employment barriers, namely research scientists, technical officers and administrative support staff. The respective unions were at one on the issue. They decided to take strike action on June 18, but this was deferred at the last moment (as was a later stop-work meeting) by reports that negotiations were in hand to restore the *status quo*. There was, though, considerable uneasiness among the staff for they had already been advised from their Head Office that "discussions are proceeding to identify the officers, employees and physical facilities that will be so transferred" (to the Department of Minerals and Energy). In a meeting on June 10, Mr Connor had left Dr Price and Mr Cameron without any doubt that he expected the transfer to occur.

Dr Price went public the following day with a press conference, and by June 16 the CSIRO Executive had briefed the Acting Minister for Science, Dr Cass, that the transfer of staff and facilities from CSIRO was "wholly unacceptable" to CSIRO. Dr Price does not find public debate easy; he is essentially a quiet person, but he showed that if scientists are slow to ignite, they can be devilishly long in burning.

Attempts by Dr Price and the CSIRO Advisory Council to see the Prime Minister failed, but Dr Cass had some effect with a well-publicised telegram to Mr Whitlam. The CSIRO Officers Association took out a writ in the High Court seeking to block the transfer on grounds of illegality related to their much prized working conditions which are independent of strict public service rules. On June 18, the 40-odd Chiefs of CSIRO Divisions had met in emergency session—always some achievement in Australia due to the distance and cost of travel to Canberra. The Chiefs unanimously (including the various Minerals Research Chiefs) telegraphed a stinging protest to Mr Whitlam. On June 20, a massive petition gathered hurriedly from 4,000 CSIRO staff, from technicians through to Chiefs and Executive, was presented to the Prime Minister's office. Six days later, the staff took out a large advertisement in the national press, timed to precede by two days the Bass byelection.

The points common to all protests included the arbitrary manner of handing down the decision without previous consultation, the value of CSIRO's unique model of organisation, its formula of freedom from political interference, and concern for morale.

Appeals to the 1974 OECD report on Australian science which recommended that CSIRO be kept intact were added to requests that the question be referred to the new Australian Science and Technology Council.

Genetic manipulation and the WHO

THE involvement of the World Health Organisation in the controversy that at present surrounds genetic manipulation experiments is welcome for a number of reasons, not all of them immediately obvious. It is true that Dr Halfdan Mahler, the WHO's Director General, has not himself made a direct statement of the position he is taking on behalf of the Organisation. But the publication of the recommendations made to him by his Advisory Committee on Medical Research (ACMR) is sufficient indication of the firm intention to become involved, the more so since one of these recommendations suggests that "the Director General pursue appropriate means" for publicising the committee's conclusions on this subject.

The hazards involved in genetic manipulation, the need to ensure that every possible precaution is taken in controlling research, and the importance of keeping the public aware and as well informed as possible, have already aroused discussion and violent debate worldwide, and such matters were already high on the agenda of this year's meeting of the ACMR. It is to that committee that the Director General would naturally turn for advice, in particular in the form of a lengthy and comprehensive statement from Dr Joshua Lederberg of Stanford University School of Medicine.

Extreme opponents of the work in this field will certainly be concerned at the very positive nature of the committee's first recommendation, which is to the effect that "The continuation (under appropriate safeguards) of microbiological research, including genetic manipulation and cell fusion studies, is of the utmost importance for progress in medicine and public health".

Other recommendations make it quite clear that the committee sees the WHO as playing a leading part at the international level, in keeping all concerned—in the scientific community as well as governmentally—continually aware of developments in this field and of the implicit dangers. On a more positive note, priority should be given to research aimed at the development of the safest possible biomaterials for such research, and to the evolution of techniques that reduce the hazards of working with them. Internally, the

WHO itself is recommended to co-ordinate all activities (at present scattered among a number of different departments) dealing with subjects in any way connected with this field. The organisation, too, will need to establish itself as "the international focus for information and its dissemination at the national level; "collaborating centres" for work in this field should be designated; and the WHO will need to play a leading part in the training and inter-country liaison that must be part of the essentially global approach to research and development of this kind. □

UNESCO science in S. E. Asia

from Yoshinobu Kakiuchi, Tokyo

AT its 17th General Conference UNESCO adopted a resolution aimed at the promotion of research and advanced training on the basic sciences with special emphasis on the needs of developing countries, and to this end UNESCO headquarters in Paris and the Field Science Office for South-East Asia in Jakarta have been working with the Japanese National Commission for UNESCO.

The meeting on regional cooperation in basic science in South-East Asia was held in Tokyo last year to consider approaches, and scientists from nine countries from South-East Asia (in-cluding Korea and Japan), and observers from Australia and New Zealand and representatives of international organisations joined the meeting. Taking the survey report prepared by two UNESCO consultants into consideration, the meeting agreed to select the field of the chemistry of natural products including microbiology as the first approach to the project.

The second meeting on regional cooperation was held in March this year in Tokyo and Osaka, and the meeting confirmed the establishment of the "Regional Network for Microbiology of Natural Products in South-East Asia" and the name of the interim points of contact of each participating country (a university involved in studies in the field) was suggested by the participant representing the country. The meeting also scheduled a meeting some time in late 1975 to discuss a more detailed picture of the similar network in chemistry, the establishment of which has already been agreed upon in the first meeting.

UNESCO is trying to mobilise all possible means available within the existing framework of its regular budget, but there is a need for more resources for this purpose. Japan has agreed to contribute cash in the form of a fund-in trust designed for use for specified objectives. There has been a fairly long experience in Japan of running UNESCO international graduate courses, one for chemistry and the other for microbiology (this is actually a one year course for foreign students, not necessarily those from Asian countries) and Osaka University is operating the course for microbiology, with the full consultation and cooperation of microbiologists from universities and research institutions all over Japan. Japanese scientists recommended Osaka University to serve as the Japanese point of contact, after consultation with Professor Kei Arima of University of Tokyo, who is at present a member of the International Cell Research Organisation (ICRO).

For the fiscal year 1975, Japan contributes $50,000 in the form of fund-in trust, and also donates approximately $18,000 equivalent of research equipment. Similar contributions will be expected in 1976. Funds and equipment are to be used exclusively for the academic activities of the network both in microbiology and chemistry, but the amount is by no means sufficient.

At the last Tokyo-Osaka meeting, Mahidol University in Bangkok, Thailand, agreed to serve as the regional centre for microbiology. It also functions as the national point of contact of Thailand, and Professor Pornchai Matangkasombut, who is in charge of research in microbiology in Mahidol University, is at present heavily involved in detailed planning of the structure and activities of the regional network. □

"It is undoubtedly a misnomer to speak of a British technological strategy". That's the harsh verdict of Dr Robert Gilpin, Professor of Public and International Affairs at Princeton, delivered in a mammoth report to a Congressional committee last month. Gilpin suggested that for various reasons, the British "on the whole have made very poor use of their rich scientific and technological resources", and he warned that the United States may make the same mistakes.

Gilpin's observations on Britain's technology policy were contained in a special report to the Joint Economic Committee on the hoary problem of how to integrate science policy and economic policy. Noting that "underlying the British malaise has been the problem of making the adjustment from the status of a global imperial power to that of a middle-sized European state", Gilpin suggested that the relative industrial decline of the United States poses similar problems to that country. The US, he suggested, should learn from the mistakes of the British.

"In the first place", Gilpin suggests, "British Government expenditures, like those of the US have been overly concentrated in a relatively few areas such as defense, space and atomic energy. The government has taken upon itself the role of entrepreneurship and has concentrated upon commercial development instead of on research, exploratory development and related activities. In substituting its judgment for that of private entrepreneurs with respect to the commercial 'ripeness' of particular high technology projects, the government has assumed a responsibility and tasks which governments do not do well. As a consequence of this neglect of the market very few of these costly projects have had commercial success".

Gilpin goes on to suggest that "the British government in a number of significant cases has made commitments to full-scale commercial development of particular technologies too early and on too big a scale" and notes that "there has been a neglect of more traditional sectors of the economy which for historical and institutional reasons tend to under-invest in R & D."

Finally, he suggests that "the British have failed to integrate sufficiently the three estates of science and technology; universities, government and industry. They have failed to create the necessary mechanisms to bring together the sources of new scientific and technical knowledge and the industrial users of knowledge. A disproportionately high fraction of British research and development has been conducted in government laboratories or in industry-wide cooperative laboratories catering to specific industrial sectors . . . While this latter set-up has served to improve the state of the several technical arts . . . the spill-over of government-supported military, and related research into the private industrial sector has been minimised".

The underlying failure of British science and technology policy, Gilpin asserts, is that "the government has tried to supplement rather than complement the private market . . . As a consequence, although the British are among the most technologically rich and resourceful people in the world, they have been unable to harness these resources to generate a sufficiently high rate of economic growth and competitive imports".

Canadian science: the golden era

from David Spurgeon, Ottawa

IN his final annual report as chairman of the Science Council of Canada, Dr Roger Gaudry looks back to a "golden era" of science policy in 1966, when the council was created, and forward another nine years to the problems of 1984. And in passing he comments on the crises of today, in which "the comfortable academic debate over science policy has largely become a thing of the past for most of us."

The former rector of the University of Montreal, who recently took over as first president of council of the new United Nations University, was the Science Council of Canada's second chairman, after Dr Omond Solandt, the founding chairman. His terms expired this summer.

The "science policy era" had barely begun when the Council was created in 1966, Dr Gaudry recalls. The Glassco Royal Commission Report on Government Organisation was barely three years old, the Senate special committee on science policy had not yet been established, and Canada had not yet been investigated in the OECD series of national science policy studies.

Before 1966, the country's science policy was, in the terminology of the Senate committee report, a "hidden" one. This, said Dr Gaudry, was because the greatest portion of policy work was not close to the point of application and thus was of interest only to the scientific community; most federally-supported science was housed in government laboratories and the universities and was largely self-determined; and because federal funds were largely given without strings, their allocations were not politically important. "Rarely did science or science-related items become public issues," says Gaudry.

By 1966, however, three sets of issues related to science and technology surfaced in public discussions and the press: the controversy over four Big Science projects; arguments over the scale and distribution of growing national research and development expenditures; and discussions over mechanisms for policy formulation and implementation.

The four Big Science projects were: Atomic Energy of Canada Limited's plea for construction of an Intense Neutron Generator (ING), which was opposed by many in the academic engineering community; the withdrawal by the federal Department of Industry of support of McGill University's High Altitude Research Project (HARP); the federal Department of Energy, Mines and Resources' plan to construct the Queen Elizabeth II Telescope in British Columbia, in the face of divided support from astronomers; and the proposal by three (later four) universities in western Canada to build a Tri-university Meson Facility (TRIUMF). The latter was the only one of the four projects destined to be funded to completion.

"Viewed in retrospect," says the Science Council chairman, "the mid-sixties were a golden era for science and technology, an era of enthusiasm, of rising budgets and of rising hopes. Canada's gross national expenditure on R & D was 15 per cent higher than in 1965, and that at a time when the annual rate of inflation was about 3.6 per cent."

Gaudry: looking back, looking forward

Science policy discussion of the era focused heavily on matters of research *per se*, as opposed to questions of the uses of science or the effects of technological programs, says Dr Gaudry. Even the Big Science projects had scientific, not technological goals. The participants were drawn almost exclusively from the scientific community itself.

By contrast, "the nature of today's discussion is both more technological and more political in character . . . many of the political issues of today revolve around what technology is, or is not, doing for us." The arguments now surround the James Bay Hydro Project, the Mackenzie Valley Pipeline, the Alberta Oil Sands Project, and the establishment of a second international airport near Toronto, at Pickering, Ont. And these arguments are over the validity of the economic objectives, the environmental impact and the costs, which are expressed in billions rather than millions of dollars.

"The adversaries in these discussions come from all segments of our society rather than from the confines of the scientific community *per se*. What has changed materially, it seems to me, is the nature of the public's attitude to technological development. Our outlook has matured considerably, we realise that 'bigger' is not necessarily 'better', we have recognised the concept of external costs which can spring as side effects from major projects, and all in all we take a more circumspect view of potential technological changes. What remains to be seen is the extent to which scientific evidence will be given due weight in decision making."

Thus the perception of what science policy is all about has changed since 1966, as well as the substantive issues. Environmentalism, anti-economic growth thought, economic nationalism, and the emergence of the provinces as significant factors in technological areas, have all come to the fore.

In the background, there has been a small but articulate "anti-science" movement, and demands for technology assessment. Debate concerning industrial strategy has also arisen. And through it all runs the thread of social responsibility, "the growing recognition that our technologies can be made either to serve or to dominate our society."

Looking ahead, Dr Gaudry sees three international problems as important: population growth and inadequate food supplies; resource supply problems due both to depletion and the action of international cartels; the possibility of nuclear proliferation.

He suggests Canada should consider participating in international technological ventures, and says she should avoid being isolated in the face of increasingly organised trade blocs. Domestically, there will have to be some form of national long-range planning in order to solve some problems, such as that of energy supply.

Changes in the structure of the population will have to be coped with, as well as chemical assaults on the environment. Industrial strife may follow technological change if not handled properly, and more public attention may have to be paid to defence technology. And "we will be forced to pay more attention to the concepts of a 'conserver society' and will move away from the maxim that any 'demand' must be satisfied, irrespective of how the demand is generated."

On the technological front, Dr Gaudry sees growing concern over increasing dependence on systems so large that redundancy in them cannot be afforded. And on the basic research front, the moral and ethical dilemmas of progress in some areas of biology, particularly genetics, will influence policy. □

A PROTOTYPE British-built rice mill, costing £10,000, is on its way to Indonesia, where the government plans to tackle inefficiency in rice milling by introducing modern mills of a sufficiently small scale to suit the needs of the country's predominantly subsistence rural economy. At present Indonesia's crop is processed largely on a small scale, either by hand pounding or by milling in wasteful machines (the most widely used being a modification of the 19th century Engleberg coffee grinder, which only produces 500 kg of edible white rice—more than half of which is broken grain—from a tonne of paddy). However, official statistics rate milling efficiency at an inflated level of 60% probably because extrapolations from a few large, modern mills are applied to the total paddy crop, although, as a 1972 UNIDO survey pointed out, these mills only handle a small percentage of the Asian paddy harvest.

Rice constitutes the staple diet of more than a third of the world's population, in particular, of 90% of the low income families in the densely populated regions of tropical Asia. Discussions of rice production are sometimes obscured by the fact that—unlike the other staples, wheat and maize, with which rice is often bracketed—there is a substantial difference between the weight of the harvested, threshed and dried paddy, often called rice, and the final yield of edible milled rice. Avoidable mechanical damage during this milling process could be destroying 20–25% of the potential yield of edible rice from paddy, according to surveys carried out in Indonesia and the Philippines by British consulting engineers and agronomists, under the auspices of the British Agricultural Export Council and the Department of Trade and Industry.

In the milling process, the inedible outer hull, which makes up about 20% by weight of the harvested dried paddy, is removed, and the final yield of edible white rice, separated from the bran—which makes up a further 10% of the paddy weight—is, ideally, 70%. Milling efficiency in the various countries of tropical Asia is said to vary between 60% and 70%, but some British experts believe, on the basis of observations in Indonesia and the

Grain gains

by Eleanor Lawrence

Philippines over the past few years, that the published figures for milling efficiency are over optimistic.

A large modern mill which deals with the paddy in several steps—hulling, separating the bran, and polishing the grain—can produce up to 700 kg of milled white rice per tonne but these large mills, capable of handling anything up to 100 tonnes of paddy an hour do not fit into the social pattern of a predominantly subsistence rural economy. The small farmer and his family, who in Indonesia, produce on average 1.5 tonnes of paddy from two crops on their half-hectare small-holding each year will eat the rice from about 1,200 kg of this, which will be milled locally or hand-pounded on the farm and will only send the surplus to the modern mills which are often far away and often inaccessible at certain times of the year.

The Indonesian government is now remedying this situation and has introduced a programme of small, more efficient local mills which can deal with the paddy at all stages from the initial threshing of wet paddy from the straw. The mechanically-dried paddy can be stored in silos until needed. These mills will incorporate a more efficient huller, which is being developed in Britain at the moment, and would be able to recover about 65% of the paddy as edible milled white rice of good quality, as well as conserving the oil and protein-rich bran for use as an animal feed and producing clean hulls which will be used to power the mechanical driers (specially converted from oil-burners). With traditional milling, either in the Engleberg mill or by hand pounding, an unusable melange of hulls, bran and powdered rice (20%, 10% and 20% respectively of the original paddy weight) is left either to smoulder or to be dumped in nearby marshy land. Each mill would serve about four square miles bringing it within easy reach of all the farmers in the area.

For Indonesia, with an annual paddy production of upwards of 23 million tonnes, a real milling efficiency of 65% covering the total paddy crop would provide an extra 3 million tonnes of edible milled rice. A preliminary survey in the Philippines has also indicated that milling efficiency over the total paddy crop is much lower than officially assumed. If the situation in Indonesia and the Philippines is paralleled throughout tropical Asia, more than 16 million tonnes of edible milled rice are being lost each year in dust, several times the entire world exports of rice each year—a substantial contribution to the 'food crisis' endemic amongst the peoples of tropical Asia who need the rice only they can produce.

A little knowledge . . .

from Vera Rich, London

" EVERYONE has won", announced Mr Brezhnev at the end of the recent Helsinki talks on detente and arms limitation. According to the rules of Lewis Carroll's caucus race, everyone should therefore have prizes. But although the agenda of the conference included not only political and economic issues, but also human rights, the free flow of information between east and west, and the possibility for all citizens to travel freely, one group at least stands to gain no benefits from the Helsinki agreement—the ever-growing group of Soviet Jewish refusniks. Hardly was the ink dry on the agreement, when the Moscow cyberneticist Aleksandr Lerner was informed "officially" that Helsinki will mean "no change" in the rules governing the emigration of Soviet Jews.

The problems involved in visa applications are particularly acute for scientists, since it is easy for the authorities to claim that they have had access to classified information. With the new atmosphere of detente, this may lead to some curious anomalies. One such case is that of Aleksandr Druk, who was working on the electronics of the space programme before he applied for a visa and was subsequently dismissed some three years ago. Although he had never visited the Baikonur cosmodrome or even seen a spacecraft, he has been informed that the question of his emigration "will be decided only in 1980". Yet, as Druk himself has pointed out, the Apollo–Soyuz mission has led to the sharing of information of far more potential importance than any which he could divulge to the West.

In other fields, too, the clamp down on Soviet Jewish scientists continues. A number of them are at present under considerable pressure because of their connection with the *samizdat* journal *Jews in the USSR*. This journal, founded by Professor Aleksandr Voronel (the founder of the "Sunday Seminars" for refusniks), carried articles on historical, philosophical and religious topics, and was intended, according to Voronel, to explore the meaning of Jewish traditions and culture against the background of tacit discrimination in which Soviet Jews must live. A number of prominent scientists were connected with the journal; those under pressure at the moment include the physicists Aleksandr Lunts and Eitan Finkel'shtein and the cyberneticist Grigorii Rosenshtein. □

IT came to the table accompanied by the limp, succulent bacon and hard toast for which English breakfasts are famous, and it was so delicious that I bought a jar to take back to the States. The label said, "Contains Sugar, Oranges, No Preservatives, No Colouring". Now sugar is, of course, one of the most ancient and universal of food preservatives, and is certainly the one used in largest quantities. The colour of oranges makes them glow like lamps against the dark green leaves in the citrus groves of California. Colourless marmalade without sugar is difficult to imagine. The label was written, of course, to quiet the fears of those who are convinced that they are being harmed by food preservatives and food colouring; that they are hapless victims of malevolent concocters of artificial viands. These fears are even voiced by the Neuberger Report which says "Flavouring agents and other food additives may have harmful effects" (p. 6) . . . "Food additives may be converted into carcinogens" (p. 138). Presumably the authors were talking about nitrites, but generalised accusations are easy to make and difficult to pin down. Rainwater during a thunderstorm contains nitrites, too.

The skin of an orange is its protection against invaders. I have no idea whether orange oil would pass all of the elaborate safety tests that are used for new food additives but I think it is quite unlikely. It contains forty-two

Peel meal
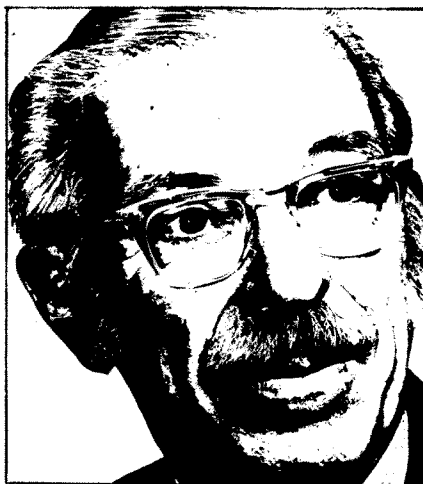
THOMAS JUKES

chemical entities including 12 alcohols, 9 aldehydes, 2 esters, 14 hydrocarbons and 4 ketones. One of the entities is a phenolic compound called tangeretin. When tangeretin was injected subcutaneously into pregnant rats at a level of 10 mg per kilo of body weight, 83% of the offspring were born dead, or died within three days. Foods are tested for toxicity, of course, by feeding rather than by injection, but one can imagine what a hullabaloo would start if a "synthetic" food additive gave such a response. There are hundreds of "natural" components of foods that

have not been evaluated for toxicity in human subjects, and probably never will be. If we believe the assurances of analytical chemists that traces of a substance are present, we must also learn to accept the findings of toxicology that there are thresholds of toxicity that can be experimentally defined. But we tend to trust Mother Nature, and suspect the organic chemist, so that "synthetics" are examined much more stringently than natural compounds. Our lives are a curious mixture of rejection and acceptance of science, and this shows prominently in attitudes towards food.

Somehow there is a feeling that human beings have developed the ability to cope with injurious substances in common foods by an evolutionary process, but that a "new" chemical is quite likely to be unmanageable. This assumption is unwarranted as a generalisation. The process of alimentation consists of devouring plants and small animals that have always contained numerous "chemicals" that are more or less toxic. These are dealt with by excretion or metabolism unless the amounts ingested become too high for comfort. So, most literally, we may have a "gut feeling" that orange marmalade is a great food; and in this I would include even the less fancy brands to which extra amounts of citric acid and pectin, two normal ingredients of oranges, have been added. □

correspondence

The waiting game

SIR,—I wish to bring to the attention of all university departments the problems of one section of the scientific community; UK postdoctoral fellows currently engaged in research at overseas institutions, which I feel will add to the current discussion of the future structure of the UK university system. Other than the ridiculous practice of setting deadlines for the receipt of applications for university positions which expire before the particular advertisement reaches foreign shores, there is a much more serious problem involving the time frame.

In my own experience university lectureships tenable from October 1975 were advertised in your columns starting in May. Allowing three months for the receipt of applications and testimonials, the short-listing of candidates and finally the acceptance by one candidate, this means that perhaps several

hundred unsuccessful applicants are kept in limbo until August to hear their fate. Amongst these the applicants from overseas are often worse off because, due to visa problems they cannot retain their fellowships indefinitely. Often such candidates will also have applied to universities in other European countries where things are decided more rapidly; this can mean being faced with the decision to accept or reject a firm offer from, say a German laboratory, by the middle of July, or risk everything and wait upon the deliberation of the ponderous UK system.

It is apparent that to ensure that UK university appointments are made from the best pool of candidates available there must be radical changes in the hiring system. Since this would require advance information of budgets and staff needs, government and university cooperation is needed.

RICHARD JAMES
Princeton University, New Jersey

Close relatives

SIR,—Gold (*Nature*, July 10, 1975) has shown how two individuals (a mother and baby) may be differentially aged by a general relativistic effect. However, the method chosen involves the mother going out to collect masses from afar, since the act of 'going out' cannot be achieved without the mother experiencing an acceleration, then the mother like the baby will also age more slowly than an inertial observer situated at a large distance from the mass shell. Indeed, during her nightly travels it is possible for the mother to age more slowly than the baby.

I therefore question whether it is at all possible for a person to construct such a mass shell without experiencing acceleration.

Yours sincerely,
L. A. KING
24 Garston Crescent,
Calcot, Reading, UK

news and views

Mercury since Mariner 10
from a Correspondent

> The First International Colloquium on Mercury was held at the California Institute of Technology on June 25–27, 1975. It was chaired by Bruce Murray of Caltech.

COMPARATIVE terrestrial planetology is an interdisciplinary field which has come of age as a result of satellite studies of the Moon, Mars and, most recently, Mercury. In the last decade, a number of complex, unmanned devices has investigated these objects and man himself has personally inspected one of them. The most remarkable result of these studies has been the identification in the photographs obtained of a universal process of cratering. The consensus is that most, if not all, craters are due to impact bombardment by planetesimals, some of which come from beyond the orbit of Uranus. Combined with the returned lunar samples, providing age dating, it now appears certain that early in the history of the formation of the Solar System, the terrestrial planets were subjected to an intense, cataclysmic bombardment process which has mainly shaped their surfaces unless they

possess substantial atmospheres. These matters and other new results concerning the innermost terrestrial planet, Mercury, were discussed and debated at the recent colloquium.

Understanding of Mercury is based primarily upon the USA Mariner 10 spacecraft results. They were obtained during an unprecedented celestial sequence of three successful fly-bys of the planet on 29 March 1974, 21 September 1974 and 16 March 1975. In view of current economic conditions, the space technology community can take great satisfaction in the multiple re-encounters achieved because they permitted a unique synergistic study of unexpected results obtained at first encounter. Any future return to Mercury seems unlikely for at least the next decade.

The most unexpected result of the first encounter was the identification of a strong solar wind interaction with the planet. This leads to the development of a detached bow shock wave as the solar plasma is deflected around the planet and the occurrence of intense bursts of energetic particles in the magnetosphere of the planet. The third fly-by unequivocally confirmed the

existence of the modest magnetic field (about 1% of Earth's intensity) and solar wind interaction. The consensus at the colloquium was that the field origin is most probably due to an active dynamo in the planetary interior. It was noted that from a magnetohydrodynamic fluid viewpoint, Mercury and its interior are rotating rapidly and thus could sustain such a dynamo.

In spite of its great success, it is doubtful if Mariner 10 will provide a precise measure of the moments of inertia and hence the distribution of mass within the planet. Nonetheless, the indirect evidence for a large, dense core seems compelling based upon theoretical models of the thermal history of the planet, global expressions of compressional features, and the existence of a modest magnetic field. The uncertainty in geochemical characteristics, especially of the radiogenic isotopes, leaves many degrees of freedom in predicting the present thermo-mechanical state of the interior. Some think that convection is inevitable in order to account for the unique synchronisation of the spin period and orbital periods of Mercury (T_{orb} = $3/2 \, T_{spin}$). One specific issue unresolved is distribution of heat sources which would be responsible for forming a large core (70% of the surface radius) and maintaining it sufficiently fluid-like to permit convection up to the present time.

One of the significant results of the high fidelity photographic reconnaissance of Mercury was the identification of heavily cratered terrains and large basins readily evident in all photographs, the largest of these being Caloris with a diameter of 1,300 km! The development of a cratering chronology was one of the principal challenges to comparative planetologists studying impact bombardment of the terrestrial planets. The higher gravity on Mercury limits the radial extent of ejecta blankets and permits a more pristine view of the cratering sequence than on the Moon, with its lower gravity, or on Mars, where alteration by Aeolian weathering occurs extensively.

The extent of volcanic processes, in both space and time, was debated as offering the most logical explanation
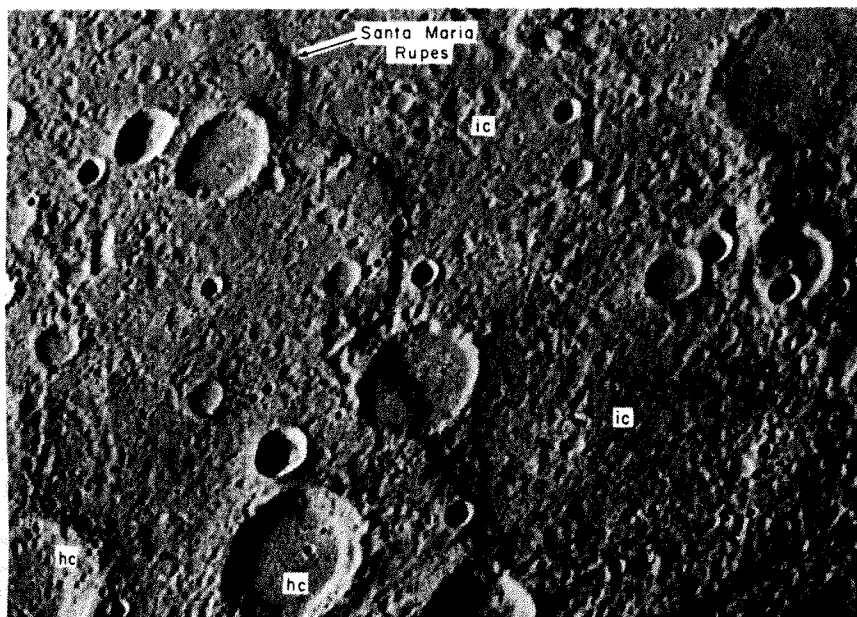
Intercrater plains (ic) and heavily cratered terrain (hc) typical of much of Mercury outside the area affected by the formation of the Caloris basin. Abundant shallow elongate craters and crater chains are present on the intercrater plains. Prominent scarp Santa Maria Rupes cuts both intercrater plains and old craters. Scene is 200 km across.

of the smooth plains, wrinkle ridges and lobate scarps observed. Unquestionably to some, the plains areas appear filled as a result of internal activity. But yet how is it possible for younger craters to be floored with plains-like looking material which the cratering process should have obviously disturbed? The view was also expressed that the intercrater plains represent a process contemporary with the cratering and occur as a result of impact melting. The debate is reminiscent of the lunar questions in the mid-1960s following the early lunar orbiter photographs but prior to the Apollo landings.

It is readily admitted that it is not yet possible to consider development of a single model of evolution of this planet. In addition to its anomalously high average density, we now know its surface to be very Moon-like and yet with its modest magnetic field its interior must be more Earth-like. Where Mercury fits in the grand scheme of the origin of the terrestrial planets is not certain. It can reasonably be expected that future studies of these results and comparisons with the Moon and Mars (with the USA's Viking 76 landers) will shed light on this fundamental question.

Human cytogenetic registries

from a Correspondent

THE astonishing success of human cytogeneticists in communicating their discoveries in a standardised and comprehensible way is due to their early recognition of the need for a standard system of nomenclature. To this end, participants at four international meetings have made recommendations and devised systems of nomenclature, each of which has extended and modified the previous one and thus successfully kept pace with rapid technical advances. The meetings were arranged on an *ad hoc* basis and had no authority save their broadly based international representation and the excellent reports which resulted. Their recommendations have been followed by virtually all workers in human cytogenetics.

The question of an international registry of abnormal human karyotypes was first raised in 1966 because many people felt that unpublished data on individuals with abnormal chromosomes (and all laboratories of human cytogenetics have such data) could, if collected and pooled, be used in some worthwhile way. The topic was first considered in depth on April 7 and 8 of this year when a standing committee on human cytogenetics, set up after the 1971 international meeting held in Paris,

sponsored a workshop in Edinburgh on human cytogenetic registries. The meeting was attended by nine individuals who run such registries, by consultants and by members of the standing committee.

The workshop first considered cytogenetic registries generally. It was agreed that it was absolutely essential that the aims and goals of a cytogenetic registry be clearly defined and that the output of useful data from such a registry was dependent upon, and limited by, the methods of ascertainment employed in the registry and by the quality and uniformity of the information within it. Subsequent to discussion of the methods of intake into a registry, the question of patient confidentiality, the rigorous quality control needed for data in the registry and the necessity for the continued surveillance of patients for certain types of registries, the participants turned their attention to the question of an international registry of abnormal human karyotypes. After considering a variety of problems to which cytogenetic registries might address themselves, the participants decided that their solution would not be helped by an international registry.

Therefore, with one dissension (Dr D. S. Borgaonkar: see *Nature*, **253**, 591; 1975) they recommended that an international cytogenetic registry should not be set up at this time and that if, at some future date, an international registry should be considered of value, it should be established only after careful planning and under the guidance of an international advisory committee. They also recommended that the establishment of regional and national cytogenetic registries should be encouraged, as should cooperation between them and existing registries. They further advised, again with one dissension, that an international advisory committee on cytogenetic registries should be set up by the standing committee of the Paris conference and, lastly, that the standing committee should advise international organisations and appropriate national agencies of these developments and solicit their cooperation and support.

The workshop report was accepted by the standing committee who will publish it later this year as part of a supplement to the Paris Nomenclature. In the light of the recommendations the standing committee has already established an advisory committee on human cytogenetic registries under the chairmanship of Dr James R. Miller, Department of Medical Genetics, University of British Columbia, Vancouver.

At a time when few regional or national registries have yet shown their worth, it seems that the Edinburgh

workshop report reflects a very sensible decision not to support at present the establishment of an international cytogenetics registry which would, in all probability, turn into a cumbersome, expensive and unproductive way of disposing of unpublished data.

Label triangulation becomes feasible

from a Correspondent

A Brookhaven Symposium on neutron scattering in biological research was held on June 2–6 at the Brookhaven National Laboratory, New York.

INVESTIGATING the topography of ribosomal proteins by the chemical methods discussed recently in *Nature* (**254**, 555; 1975) can only lead to a qualitative model of a ribosome. The standard methods for structure determination are diffraction methods. At the symposium two groups showed independently that the application of the 'label triangulation method' to ribosomes has become an experimental reality. This method is in principle capable of giving the complete quaternary structure of the 54 proteins in a ribosome in a quantitative and unambiguous way. It is based on low-angle scattering in solution and was first proposed for X rays (Hoppe, *Israel J. Chem.*, **10**, 321; 1972) and shortly thereafter for neutrons (Engelman and Moore, *Proc. natn. Acad. Sci. U.S.A.*, **69**, 1997; 1972).

Scattering in solutions can be treated like gas diffraction. Using the corresponding theory it can be shown that the linear combination of four scattering curves $u = a + b - c - d$ (scattering of solutions of equal amounts of a, the molecular complex to be studied; b, the molecular complex labelled with two additional scatterers A and B; c, the molecular complex labelled with A only; d, the molecular complex labelled with B) corresponds to a damped sine curve with a wavelength inversely proportional to the distance AB (see also Kratky *et al.*, *Mh. Chem.*, **76**, 281; 1946; **78**, 295; 1948). A systematic labelling is possible by disaggregation of the ribosome (respectively of the 30S or 50S subunit) and by reaggregation, replacing unlabelled proteins by labelled proteins. In the case of X rays labelling can be done by introducing heavy atoms; in case of neutrons by replacing deuterium (in a completely deuterated subunit) with hydrogen. A triangulation of the distances leads to the quaternary structure. Approximately 200 distances can be measured in the 30S subunit and 500 in the 50S subunit.

It is evident that not only a considerable amount of diffraction work but also a great amount of biochemical work is necessary for a triangulation.

The difference scattering curve u=a+b—c—d can be measured in two different ways· one can prepare and measure four solutions of the derivatives (as proposed by Engelman and Moore, 1972) or one can prepare two mixed solutions, which deliver respectively a+b and c+d. It was shown in the X-ray triangulation paper (Hoppe, 1972), that in the latter case all inter-particle effects (even if stemming from aggregations) will be cancelled (see also Hoppe, *J. molec. Biol.*, **78**, 581, 1973). This is a very unusual situation in solution scattering. It is easy to see how important—even essential—this cancelling is for the label distance measurement in such a giant assembly as the ribosome. The averaged label concentration in a 50S subunit is only 1%. One is therefore forced to work on highly concentrated solutions (11% of the 50S subunit and more than 20% of the 30S subunit in the later mentioned measurements). Moreover, the additional determination of the shapes of the proteins in neutron work—which requires accurate scattering curves—would be entirely impossible.

The experimental results reported in Brookhaven have been achieved on the protein pairs S3–S7 (115 Å), S2–S5 (104 Å), S5–S8 (38 Å) in the 30S subunit (D. M Engelman, P. B. Moore, B. P. Schoenborn, and collaborators) on the Brookhaven reactor and on L7/L12–L10 (100 Å), (W. Hoppe, H. G. Wittmann, H. L. Crespi, and collaborators) on the Grenoble reactor. In the latter case the two proteins L7/L12 are identical—except for a N-terminal group in L12. It has been deduced from other experiments that the pair L7/L12 should be a dimer. It can therefore be treated as a single protein. Both groups have used the mixture method; both labelled by replacement of hydrogen with deuterium (instead of *vice versa* as originally proposed in the neutron paper) in order to economise on deuterated material. The intensity differences are very small (with an order of magnitude of ~ 1%) and could only be measured using low angle diffraction matrix instruments as first developed in Grenoble by K. Ibel.

Replacement of hydrogen by deuterium was first used in membrane work (Kirschner and Caspar, *Ann. N.Y. Acad. Sci*, **195**, (ser. 5), 309) and it is therefore not surprising that much effort was reported in Brookhaven to use this type of isomorphous replacement not only for myelin membranes (D. Kirschner, University of Basle, and D. L. D. Caspar, Brandeis University) but also for other biological systems:

for example M. J. Yeager (Yale University) and M. Chabre, retina; V. Luzzati (CNRS, Gif-sur-Yvette), lipoproteins; A. Miller (University of Oxford), collagen, E. M. Bradbury (Portsmouth Polytechnic), chromatin; J. Randall (University of Edinburgh), catalase. All work has been done by exchange of water or of hydrogen ions by respectively heavy water or deuterium ions. It is evident that the non-exchangeable replacement of core hydrogen by growth of microorganisms (as used in the triangulation work) will open up new possibilities. It is therefore of great practical importance that *E. coli* with nearly fully deuterated proteins can grow from hydrogenated substrates like glucose (P. Moore). Interesting applications of direct methods were reported, originating from earlier work of Hosemann and Bagchi (see Matter, *Direct Analysis of Diffraction*, Amsterdam, 1962). Of further interest is the fact that the first projects were aimed at the use of neutron spectroscopy in biological systems (J. White and W. L. Peticolas). A report on the Brookhaven Symposium would be incomplete without mention of the elegant work on protein crystal structure determination of haem proteins (B. P. Schoenborn, Brookhaven National Laboratory).

Tektites and their origins

from David W. Hughes

TEKTITES are small glassy objects which are found in certain localised areas on the Earth's surface. They are one of the enigmas of science, a cuckoo in a nest of meteorites.

There is still considerable doubt as to whether tektites are meteorites because they have never been seen to fall to ground. Meteorites are found randomly over the whole Earth whereas tektites only occur in certain areas, all at relatively low latitudes: the southern half of the Australian continent, Indomalaysia, the Ivory Coast in Africa, Czechoslovakia and North America (Texas, Georgia and Martha's Vineyard). They do not arrive at Earth continuously either: the separate arrival events all occurred during the last 34 Myr.

About 650,000 tektites had been discovered by 1960. The Czechoslovakian Moldavites are dark green whereas all the others are jet black, their surfaces usually having a dull lustre due to abrasion and weathering. The glass is isotropic and brittle with a refractive index between 1.48 and 1.52 and a density around 2.4 g cm^{-3}. Tektites have weights up to 300 g and shapes varying from spheroidal to pear-, lens- and disk-shaped. Many of them show ablation

features and contorted flow structures as if their surfaces had been heated while travelling at high speed through a gaseous atmosphere and subsequently quickly cooled. The chemical composition is that of a silicic igneous rock with high SiO_2 (70–80%) and moderate Al_2O_3 (11–15%), the composition being relatively independent of the formation with which the tektites are associated. There are, however, distinct minor variations between the different groups and also systematic minor variations across the individual strewn fields. Tektites also contain minute shred-like inclusion of pure silica glass (lechatelierite) which can only form at temperatures in excess of 1,710 °C. Tektite composition is quite different from that of any known meteorite. The age of solidification of the primary object was found to be close to 400 Myr by using $^{87}Rb/^{87}Sr$ decay, and measuring the $^{87}Sr/^{86}Sr$ ratio. The range of values obtained is much smaller than that found for terrestrial igneous rocks and there are only slight differences between specimens from different localities. By using the $^{40}K/^{40}Ar$ ratio, the age of solidification of the glass during ablation can be found. This is equivalent to the age of arrival on Earth and varies considerably between locations, being 0.61 Myr for Australites, 1.3 Myr for Ivory Coast tektites, 14.7 Myr from Moldavites and 34 Myr for American Bediasites.

The origin of tektites is still unknown Theories can be divided into two main categories. terrestrial and lunar. One of the favoured terrestrial theories is that tektites were formed by the fusion of terrestrial material during the impact of giant meteorites. These meteorites also produced large craters in the Earth's surface such as the Ries Kessel structure in West Germany, 250 km away from the Moldavite strewn field, and the Bosumtwi crater in Ghana. The potassium–argon ages of the tektites and of the cryptic glass from around these craters agree surprisingly closely. However, these impactite glasses found near the craters are markedly different from tektites, being slaggy and porous and having abundant inclusion of partly fused material; also the problems of moving the tektite a distance of 200–300 km against air resistance and accounting for the 10^3 difference in H_2O content between terrestrial rocks and tektites are not trivial. The fact that large impacts have occurred throughout the history of the Earth and not just in the last 34 Myr must also be explained.

Another terrestrial theory advocates a crypto-volcanic origin McCall (*Meteorites and their Origins*, David and Charles; 1973) states that Reis Kessel and Bosumtwi might be extinct volcanoes and not impact craters, the

A hundred years ago

Mirage on Snowdon

On Monday, July 12, I, with a party, ascended Snowdon. The atmosphere was clear until we had reached within half a mile of the summit, when a light cloud rising stealthily from amongst the southern peaks enveloped it. Drifting towards us, when very near, the cloud dropped over the eastern shoulder of the mountain just where it dips towards Capel Curig. As we stood watching, great was our surprise and delight as we beheld painted upon it, not the *arc-en-ciel* with which we are familiar, but a complete and brilliant prismatic circle, apparently about thirty feet in diameter, in the very centre of which we ourselves were depicted, the image being somewhat enlarged but clearly defined; as we arranged the party in groups, or bowed to each other, every form and movement was faithfully reproduced in the picture. It was now about 8 o'clock, with the sun nearly in a line with us. Our guide, who had made some hundreds of ascents, had never witnessed such a sight before.

H. J. WETENHALL
from *Nature*, 12, 292; August 12, 1875

tektites being produced by fusion between volcanic emanations and country rocks. A simpler theory postulates that tektites are shed melts derived from ablating meteorites. But tektites are acidic and all known meteorites are basic.

Lunar theories propose that the tektites are ejected from the Moon in a molten form during the formation of craters by meteorite impact and during periods of lunar volcanic activity. The composition of tektites is hard to account for as most of the material brought back from the Moon has been basaltic rather than acidic. The fact that there are only four age groups of tektites, although the hundreds of thousands of lunar craters were created at many different times, counts against the impact ejecta theory. Also impact craters mainly eject matter at low angles.

Lunar volcanic ejecta as a source of tektites is strongly advocated in the recent paper of Cameron and Lowrey in *The Moon* (12, 331; 1975). Reliance on a volcanic ejection process can explain the rarity of tektite falls on Earth and their composition. The velocity of escape they need to leave the Moon requires the volcanism to be of the explosive type which terrestrially is usually associated with acidic suites. (Acidic lavas, which have very high viscosities of 10^{12}–10^{13} poise, can sustain very high pressures before disruption. This leads to massive rock and lava ejection, as with Krakatoa, which ejected 18 km^3.) As the youngest tektite is only 0.6 Myr old, lunar volcanic activity must have been very recent—and the authors tie this in with observations of lunar transient phenomena—mild degassing observed from time to time around certain craters. The lack of H_2O in lunar samples is also echoed in tektites.

Tektites ejected vertically from the Moon at a speed of 3 km s^{-1} will only reach the Earth if they start from a small area on the eastern hemisphere within 6° of the lunar equator. The orbits are hyperbolic with respect to the Earth–Moon system so if the tektite misses the Earth on the first pass it goes into a heliocentric orbit. In searching for the exact point of origin Cameron and Lowrey regard 'lunar transient phenomena' sites within the specified boundaries as prime suspects, provided, however, that other evidence of volcanic activity can be found in their vicinity. The sites are all ray craters—Censorinus, Taruntius, Messier and Messier A (the old Pickering) and it is postulated that the rays are whitened exposed rocks which have had their dust covering blown off by gases emanating from the craters. Censorinus AB has a central mound with a funnel-shaped crater similar to those of terrestrial diatremes, fumaroles and explosion pits, all manifestations of volcanism. The hummocky bands in Messier, some of which have summit craters, are also pointers towards volcanism. Steep sloped ridges inside Taruntius indicate that volcanism could have post-dated the impact explosion.

The source of tektites must lie deep within the Moon because tektites differentiated in the last 10^9 years when the Moon's heat was confined to very deep regions. Cameron and Lowrey postulate that diatremes coming from these depths discharged their ejecta vertically; and that craters such as Messier were formed by impact and then suffered volcanism because the craters were formed on mare surfaces, the scenes of original volcanic flows.

So old lunar volcanoes, the present day sites of transient lunar phenomena, provided they are in the right area of the Moon, can eject tektites which will hit the Earth. But why have these only erupted in the last 34 Myr? Why are the tektites so tightly bunched in space that they only produce small strew fields on Earth? How does such pure differentiated material get produced on the Moon? Where are all the tektites that missed the Earth the first time, only to fall to Earth an indeterminate time later? Many questions remain unanswered; these enigmatic tektites have been bothering scientists since the eighteenth century and I am sure will continue to do so long into the future.

Far infrared astronomy

from M. Rowan-Robinson

The first international conference devoted exclusively to far infrared astronomy was held at Cumberland Lodge, Windsor Great Park on July 9–11. It was organised by Queen Mary College, London, and sponsored by the Royal Astronomical Society.

THE far infrared is usually taken to mean the wavelength range 30 μm to 1 mm which, being almost inaccessible from the ground, is observed from aeroplanes, balloon platforms or (for the future) satellites. One of the most interesting discussions was of the relative merits of NASA's proposed Large Space Telescope (LST), a satellite-borne 60-cm telescope which will operate in the optical and ultraviolet as well as the infrared (plans were reported at the meeting by D. E. Kleinmann of Harvard), and balloon-borne telescopes working at an altitude above 30 km. One advantage of balloon-borne systems is that they are already in action. The University College, London group reported the latest results of their broad-band (40–300 μm) survey of the galactic plane and maps of some individual sources, while G. G. Fazio of Harvard described 70 μm studies of several galactic HII regions and some external galaxies. K. Shivandan of Naval Research Laboratories, Washington, described multicolour photometry of the Orion nebula, and several groups described interferometer systems to be flown on aircraft or balloons giving detailed spectra of strong sources in the far infrared.

Another area of outstanding interest was the microwave background, with reports of Queen Mary College's balloon-borne interferometric observations in the range 0.7–2 mm and of Leeds's forthcoming experiment. Although the Berkeley group, who have recently successfully repeated the experiment, were not represented, much of the discussion centred on the conflicting atmospheric measurements and interpretation of the Queen Mary College and Berkeley groups. The issue remained unresolved, partly due to ignorance about the amount of ozone at altitudes above 30 km, and it is to be hoped that the Leeds experiment this summer will help resolve the issue. However both groups agree on the major issue, that the cosmic background spectrum turns over shortwards

of 1 mm as expected if it is the 2.7 K black-body relic of the fireball phase of a big bang Universe. The efforts of J. V. Narlikar (Tata Institute, Bombay) to explain this background as starlight thermalised by large graphite 'whiskers' ran into heavy criticism.

Ground-based astronomy had its moments too, with F. F. Gardner of CSIRO, Australia, L. T. Little of the University of Kent, and A. Gillespie of QMC reporting on molecular line studies (formaldehyde, H_2O and CO respectively) and P. E. Clegg reporting on Queen Mary College's 1 mm continuum studies of galactic sources, galaxies and QSOs.

The theoreticians produced a variety of models of dust-clouds and HII regions, disagreeing wildly over the composition, shape and size of the dust grains, ranging from ice and silicates to formaldehyde polymers, from spheres to whiskers and snowflakes, and from 0.1 μm to 1 mm in size. Clearly much work remains for both observers and theoreticians in this exciting field.

Rock fabrics

from Robert W. Cahn

THE British Standard Conference is organised by a learned or professional society, lasts 2.5 days, has two parallel sessions, costs £25 in registration fees alone, involves a pricey conference dinner, is attended mostly by academics and industrial scientists whose institutions are still willing to pay up, and evokes a British Standard Response to the effect that, with luck, just one paper will be really interesting to the British Standard Conferee. I claim a little artistic licence—the British Standards Institution has not yet, so far as I know, laid down a standard for the BSC—but all seasoned scientists will recognise the gestalt.

Some weeks ago, in mid-May, I had the good fortune to take part in a very different kind of conference in the Geology Department at Imperial College, London. It lasted one day, cost £0.50 to attend, had a single session, and was organised entirely by a graduate student, K. McClay, on behalf of a group of graduate students and university staff (the Tectonic Studies Group); the audience consisted of a sprinkling of British and foreign academics and a lot of students, post- and undergraduate alike. The topic was "Fabrics and Textures in Rocks".

The term 'fabric' in a petrographic context denotes both preferred orientations in populations of crystal grains (what metallurgists term 'texture') and the shapes and mutual dispositions of matrix grains and subsidiary phases (what metallurgists term 'morphology').

Texture arises from mechanical deformation of rocks under pressure and resultant recrystallisation when the temperature is high enough. The processes involved must be related to those that generate deformation textures and annealing textures in metals; the Tectonics Group evidently recognise this, because the two opening speakers (I. L. Dillamore of the British Steel Corporation and R. W Cahn of Sussex University) were invited to outline the present state of metallurgical knowledge on the two kinds of textures. The geologists present showed an impressive familiarity with the mathematical techniques used to interpret the genesis of deformation textures or the processes that arise during annealing of deformed metals. Thus G. Lister of the Geological Institute, Leiden, dealt with deformation textures in quartzite and S. White of Imperial College with recrystallisation mechanisms in the same rock, both drawing extensive metallurgical parallels.

Other contributors (E. Rutter and W. Shaw of Imperial College and R. G. C. Bathurst of Liverpool University) reviewed textures and fabrics of limestones, dolomites and marbles. D. J. Barber and H. R. Wenk (Essex University) applied electron microscopy to these rocks; this technique has only recently proved feasible for petrography, following the introduction of the ion-beam thinning technique, and it seems likely to prove as fruitful here as it has done in metallurgy.

The petrographer has the advantage over the metallurgist that he can examine his (optically anisotropic) minerals by transmitted polarised light, with the aid of a universal stage. Students of quartzite use this approach to determine a 'partial texture', that is, the distribution of the orientations of *c* axes both from one grain to another, and between different parts of the same grain : in this way, detailed textural and morphological information on a population of over 100 grains can be obtained within a day's work—information that would take a metallurgist, working with X rays and optical microscopy, or with electron microscopy, many weeks to assemble. Dr White in particular, using the *c*-axis technique, was able to draw on metallurgical experience to prove the close similarity of the processes that generate high-temperature textures in quartzite.

The remaining speakers, from Leeds and Leiden, dealt with more complex minerals, such as slates and peridotites. Here again, metallurgical processes such as strain-induced grain-boundary migration were identified.

One of the quartzite specialists spoke a few weeks later at a British Standard

Conference (good of its type!), devoted to textures in metals : to a witness who was able to compare the two occasions, it was plain that the geologists were much more ready to learn from metallurgical insights than metallurgists were willing to interest themselves in geologists' problems and techniques. This is a pity, because the two groups have much to contribute to each other.

Nuclear pores

from J. R. Tata

ONE of the many mysteries surrounding the organisation and function of the cell nucleus is how this organelle maintains concentrations of ions and small molecules (especially nucleotides) higher than those in the surrounding cytoplasm, while, at the same time allowing the large ribonucleoprotein precursors of polyribosomes to pass into the cytoplasm. The characteristics of the double nuclear envelope are such that some specialised site at the nuclear surface must regulate the nucleo–cytoplasmic transactions. A likely candidate for this function is the 'nuclear pore complex' (see Franke and Scheer in *The Cell Nucleus*, vol. 1, 219, 1974 for an excellent review). Although cytologists have for a long time known about the existence of pores on the surface of the nucleus, virtually nothing is known about their chemical nature and function.

Ultrastructurally, the size (diameter 600 ± 150 Å), but not the number, of nuclear pores is remarkably constant in all eukaryotic cells. Franke's group have done considerable work in defining optimal conditions for observing these structures by electron microscopy of intact cells and of isolated nuclei and nuclear membrane preparations. A well known ultrastructural feature of the nuclear pore is the 'annulus' of non-membranous material surrounding both the inner and outer rims of the pore (Callan and Tomlin, *Proc. R. Soc.*, B137, 367; 1950; Afzelius, *Expl Cell Res.*, 8, 147; 1955). What is particularly interesting about the annulus is its characteristic eightfold symmetry arising from eight 'annular granules' with diameters of 100–250 Å (Roberts and Northcote, *Nature*, 228, 385; 1970; Franke and Scheer, *J. ultrastruct. Res.*, 30, 288; 1970). The space within the pore is often seen to be filled with varying amounts of an 'amorphous' material as well as fibrils of about 50 Å diameter, themselves sometimes studded with smaller granules of 50–100 Å. Not surprisingly, several models for the structure of this complex ensemble have been proposed.

Blobel's group at the Rockefeller

University have undertaken to isolate and characterise the nuclear pore complex as a prerequisite for exploring its function. Recently, Aaronson and Blobel (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 1007; 1975) have succeeded in isolating the nuclear pore complex of rat liver nuclei and giving a preliminary account of the composition and structure of this isolated subnuclear fraction. The procedure involves, first, the preparation of nuclear envelope, not by a method based on treatment with high concentrations of Mg^{2+} as previously used in Blobel's laboratory (Monneron, Blobel and Palade, *J. Cell Biol.*, **55**, 104; 1972), but one based on the use of DNase I (Kay, Fraser and Johnston, *Eur. J. Biochem.*, **30**, 145; 1972). The isolated nuclear membranes are then treated with detergent (Triton X–100) to solubilise phospholipid and remove the membraneous components; any residual chromatin is removed by precipitation of the nuclear pore complex with 0.3 M $MgCl_2$.

When examined in the electron microscope, Aaronson and Blobel's isolated nuclear pores were found to be associated with stretches of the peripheral lamina of the nucleus. The latter structure, which is distinct from the inner nuclear envelope, has been previously described in intact nuclei from other sources but not from rat liver (Fawcett, *Am. J. Anat.*, **119**, 129; 1966; Kalifat, Bouteille and Delarme, *J. Microsc.*, **6**, 1019; 1967). The lamina in the isolated preparation is 150 Å thick and connected to well preserved pores of diameter 700–900 Å whose lateral profile is 650 Å in diameter and 'goblet-shaped'. An interesting feature of these isolated structures is the absence of any membraneous material, which confirms an earlier suggestion by Aaronson and Blobel (*J. Cell Biol.*, **62**, 746; 1973), based on the preparation of 'nuclear ghosts', that the membrane of the double nuclear envelope is not essential for imposing or maintaining the structure of the pore complex. As regards its composition, the isolated rat liver nuclear pore complex is almost 94% protein with traces of RNA and DNA but no detectable phospholipid. The sugar content has not been determined and it would be interesting to know whether any glycoproteins are present. Electrophoresis on sodium dodecyl sulphate–polyacrylamide gels resolved the proteins of the isolated nuclear pore complex associated with the lamina into three major components of molecular weights 66,000, 68,000 and 69,000 with a minor component of much higher molecular weight. This simplicity of protein composition is, indeed, surprising in view of the complex structural elements of the nuclear pore.

What could be the functions of the nuclear pore complex in the intact cell? That the number of pores per nucleus is not constant, but is directly related to the rate of protein synthesis or ribosome accumulation (as during amphibian oogenesis), suggests a role in the nucleo–cytoplasmic transport of ribonucleoprotein particles. Isolated nuclear pore complexes do not offer any advantage in studying such a transport function, which can best be tackled by a combination of indirect methods of cell biology and cytology. But the advance made by Aaronson and Blobel offers the possibility of directly testing the role of the pore complex in carrying out terminal modification step(s) in the biogenesis of messenger and ribosomal RNA and the assembly of corresponding ribonucleoprotein particles. Thus, it would be interesting to see if the isolated nuclear pore complex has enzyme(s) for specific cleavage, polyadenylation or methylation of RNA, or whether it could somehow facilitate the terminal addition of protein(s) to 'immature' ribonucleoprotein particles just prior to their release into the cytoplasm. However, the extremely simple protein composition of the isolated nuclear pore complexes makes it likely that they retain few, if any, of such activities as they may possess in the intact cell.

Links between core and plates

from Peter J. Smith

IN a series of articles published about a decade ago, Wilson and his colleagues (see, for example, Wilson and Haggerty, *Endeavour*, **25**, 104; 1966) reported that they had established a statistical correlation between magnetic polarity and the degree of oxidation of the iron–titanium oxides in a wide variety of basalts Specifically, they showed that the higher the average oxidation state of the oxides within a basalt, the greater was the chance of the rock having reverse magnetisation. This result was, and remains, a mystery. The magnetic polarity of a rock depends on the polarity of the geomagnetic field at the time the rock formed; and the polarity and polarity changes of the field are presumably governed by processes in the Earth's core. Lava flows, on the other hand, originate in the upper mantle. The Wilson correlation thus seemed to imply an unlikely connection between core and upper mantle processes.

Since then, the so-called 'oxidation state–polarity paradox' has been largely ignored, partly because some workers have disputed its validity and partly because even those who accept the cor-

relation have been unable to decide whether or not it is significant enough to follow up. But the question of links between core and mantle was later revived by Hide and Malin (*Nature*, **225**, 605; 1970) who found a significant correlation between the Earth's gravitational field and the non-dipole geomagnetic field. This they attributed to undulations of the core–mantle interface, which would presumably influence, and be influenced by, both core and mantle motions. It also seemed to bear out in part Hide's (*Science*, **157**, 55; 1967) earlier prediction that "it would not be surprising to find that 'reversals' are correlated to some extent with other phenomena that may be affected by motions in the mantle, such as tectonic activity (mountain building, ocean-floor spreading, continental drift, and so forth) . . .".

The conceptual difficulty raised by the Wilson correlation of the mid-1960s had thus given way by 1970 to one possible physical model whereby core–mantle interaction could be achieved; since then other connections between mantle and core processes have been proposed. Won and Kuo (*J. Geophys. Res.*, **78**, 905; 1973), for example, have suggested that great earthquakes could cause the solid inner core to oscillate. Any such oscillation could be expected to influence fluid motions in the outer core and hence affect the apparently delicate mechanism of field reversal. Closer to the Earth's surface, on the other hand, the exciting earthquakes are intimately related to plate tectonic processes. The Won–Kuo hypothesis thus implies that correlations between geomagnetic field behaviour and events in the crust and upper mantle are not only possible but probable.

The near-surface phenomena envisaged here are altogether more conspicuous—Vogt (*Earth planet. Sci. Lett.*, **25**, 313; 1975) calls them 'first order' events—than the previous polarity–oxidation and gravity–magnetic field correlations. But the motion of lithospheric plates is also complex, making it difficult to define a general plate tectonic 'index' with which to compare geomagnetic behaviour. Any attempt at a general worldwide comparison is thus out of the question for the time being. As an alternative, however, Vogt has examined the four principal plates for particular examples of correlations between plate tectonic activity and geomagnetic phenomena, and has discovered enough of them to suggest that core–upper mantle coupling is real.

The first comes from the Hawaiian–Emperor chain of volcanic islands in the Pacific—a chain which comprises two more or less linear sections joining at an angle. Because these islands

increase in age from one end of the chain to the other, they have been attributed to the motion of the Pacific plate over a stationary hot spot, possibly related to a mantle plume (although other explanations are possible). The bend in the chain is then thought to be due to a sudden change in the direction of Pacific motion which can be dated from the monotonic age pattern of the islands at 42–45 Myr. But the geomagnetic reversal frequency also changed significantly at that time. Thus within the errors of dating, the only conspicuous changes in Pacific plate motion and reversal frequency during the last 70–80 million years coincide Moreover, other tectonic changes took place at the same time in other parts of the world. In the Reykjanes ridge area south of Iceland, for example, the 45 Myr isochron marks the largest change in the direction of motion between Greenland and Europe, a change which gave rise to the generation of numerous small transform faults

The second magnetic event examined by Vogt was the ending 70–77 Myr ago of the Mercanton interval, a 30–40 Myr period during which there were few or no reversals At this time, seafloor spreading first began south of the Rockall Bank in the northeast Atlantic and the Meteor and Corner Rise seamount groups were formed. In addition, there was a major change in the trend of the fracture zones in the central North Atlantic, a reflection of what appears to be the most prominent change in spreading direction between North America and Africa during the whole 180 Myr existence of this ocean basin Other major plate tectonic changes also took place about 115 Myr ago when the Mercanton interval began and the earlier Keathley sequence of reversals (150–115 Myr) ended

But if these correlations are manifestations of core–mantle coupling, the problem of precisely how the coupling occurs remains. Vogt clearly favours a return to the idea of mantle-wide convection, the most obvious and traditional physical link between the core–mantle boundary and the near-surface processes In this case, the geometry of the convection would change from time to time, thereby directly influencing the plate motions above and at the same time changing the boundary conditions at the core–mantle interface and hence the reversal frequency within the core. Whole-mantle convection has not received much support in recent years. Since Wilson's basalt correlations (which are not mentioned by Vogt) were discovered, however, an alternative form of mantle-wide motion has been proposed in the form of mantle plumes. These would seem to offer a connection between core and

plates which is both more direct and perhaps more attractive to today's earth scientists.

Convergence of information in thermal pathways

from Shin-Ho Chung

NEUROPHYSIOLOGICAL studies of different sensory systems have shown that incoming information undergoes drastic modification as it proceeds along the pathways of the nervous system, the degree of modification increasing as messages proceed centrally. Such hierachical organisation is a general principle upon which all the nervous system is constructed, and understanding the transformation of information at each level has been the dominant preoccupation of sensory neurophysiologists over the past two decades. Two recent articles by Hellon, Hensel and Schafer (*J Physiol, Lond.,* **248**, 349–357, 1975) and Hellon and Mitchell (*ibid,* 359–376) describe the successive steps taken by the nervous system in processing the information generated by thermoreceptors.

Primary sensory fibres arising from 'cold' and 'warm' receptors in the rat scrotum show action spectra resembling bell-shaped curves when frequency of impulses is plotted against scrotal temperature. The size of each receptive field is about 1 mm². These inputs are integrated by cells in the spinal cord; however, cells in the cord no longer have spot-like receptive fields but are sensitive to temperature changes over the entire extent of the scrotum Alteration of the response pattern is most clearly seen in the behaviour of cells in the thalamus. Unlike the bell-shaped action spectra of primary afferents, thalamic cells exhibit an on–off pattern of firing (see figure). A cell remains silent until a critical temperature is sensed, after which it fires at its maximal discharge frequency.
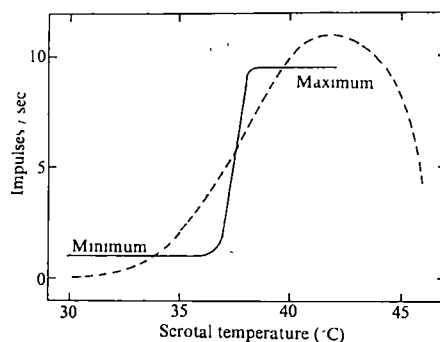
Action spectra of 'warm' receptors in the rat scrotum (broken line) and of thalamic neurones (continuous line). From Hellon and Mitchell, *J. Physiol., Lond.,* **248**, 349–357; 1975.

Different cells in the thalamus have different critical temperatures (Hellon and Misra, *J Physiol, Lond.,* **232**, 389, 1973) Thus, at the expense of spatial discrimination, activities of primary afferents are integrated by the central nervous system and, as thermal assault on the testes intensifies, more neurones are recruited in their defence. This is a sensible physiological arrangement, for the ability to discriminate which side of the scrotum is warmer is unlikely to serve any useful biological purpose

Language understanding by computer

from Arnold G Smith

A conference at MIT on June 10–13 entitled "Theoretical Issues in Natural Language Processing" was aimed at scientists working on computer models of language either in order to study the human mind, or with the more pragmatic goal of "teaching computers to speak English"

ATTEMPTS to create computer systems which understand English raise issues which reach deeply into linguistics, computer science, psychology and even philosophy. Earlier work in this field led to the realisation that a reasonable model of language comprehension would require the active use of a potentially vast amount of 'common sense'. Common sense is called upon continually though often unconsciously in making sense of what we hear, but how does one design a computer program to function analogously, organising a large corpus of knowledge so as to have the appropriate facts available at the appropriate moment? Marvin Minsky's concept of frames is the currently fashionable approach to answering this question, although it generated a great deal of controversy at the meeting.

Minsky (from MIT) was there to explain his ideas. He uses the term 'frame' basically in the sense of frame of reference, in the light of which one makes sense of a sentence, a story or a scene. An intelligent person presumably has a very large number of such frames stored away in a richly interconnected structure. At any point one of these frames is in charge of interpreting and interacting with the current environment. It has its own procedures which look for evidence to confirm its relevance to the situation at hand and, when something turns up which is inconsistent with or beyond the scope of the current frame, it has

links to likely alternative frames which will have a good chance of making sense of the input. Each frame has slots for the kinds of information it expects to find and, until these slots are filled, they are likely to have default assignments. My frame for bedrooms, which will be evoked if you start to tell me what happened in your bedroom, has defaults for things like the size of the room, and the style of the bed. In the absence of specific information, I will simply assume the presence of a normal bed to supply the default. If you then say that ten people slept in your bed last night, I will expect them to have been very crowded. On the other hand, an earlier description of your particularly huge bed would have displaced the default and so have led to different inferences. As long as a particular frame is in charge, it coordinates the information that comes in, making inferences to connect what might otherwise look like unconnected bits of evidence, and calling on other frames with narrower expertise to work with details of the situation. In this way, frames supply an interpretive context and constitute their owner's model of the world

Some critics regard the frame theory as a re-statement of ideas long current in philosophy and psychology. In fact Minsky explicitly disavows claims of formal precision for his theory, but it is oriented specifically towards computer models of cognition. Terry Winograd of Stanford pointed out various ways in which frames were suggestive of, but not merely equivalent to, a variety of other paradigms from artificial intelligence—scripts, patterns, decision trees, semantic nets, actors, and production systems. He suggested that frames share important characteristics with each of these, but that you miss part of the point of frames by limiting your perspective to any one. I see the frames concept not as a unified theory but as a vantage point for discussing the shortcomings of present systems and pointing to some of the properties that better cognitive models must have.

A workshop session on methodological issues considered the difficult problem of evaluating and comparing the contributions of different projects in the field. Two factors seem to exacerbate the situation. One is the notorious lack of perspicuity of large computer programs. The other is the fact that language is so inextricably involved with everything we do that it is hard to gain the perspective needed to see how a particular piece of work fits into the whole domain. W. A. Woods, of Bolt, Beranek and Newman, presented a particularly, well-reasoned paper on this subject. He argued,

among other things, the need for cognitively efficient representations—models whose explicit structure can act as a guide to understanding the theory they incorporate.

There was somewhat less than one might have expected in the way of new results reported at the meeting. Several people were in the process of working out some of the implications of their version of the frames idea. Roger Schank and his colleagues at Yale, for example, described initial work with a program which knows what to expect when it hears a story about someone going to a restaurant. A few sentences relating John's experience eating a lobster dinner will trigger off a variety of inferences about who cooked the lobster, how it arrived at John's table, that if he gave the waiter a large tip it was probably because he enjoyed the lobster, and so on. And Sheldon Klein, of the University of Wisconsin, passed out copies of a seven page murder story generated by his computer in nineteen seconds! But there was more emphasis on what people had been thinking about methods and paradigms than on what they had learned about language.

Motion of muscle proteins

from a Correspondent

THE dynamic behaviour of proteins—their rotation and internal flexibility—are important for many aspects of their biological function. This is particularly true in the actomyosin system of muscle, where these phenomena are intimately related to the mechanism of contraction.

In such a situation, it is obviously necessary to be able to study the behaviour of individual components of the system, and in practice this means they must be labelled in some way. Two such labelling techniques have been widely used over the last few years. In the first, the protein is labelled with a strongly fluorescent group; the time course of the decay of the anisotropy of the fluorescence after excitation with a short flash of polarised light can then yield a value for the rotational correlation time, τ_c, of the label. The second approach, known as spin-labelling, involves the attachment of a stable nitroxide free radical to the protein. The value of τ_c is obtained by careful analysis of the shape of the electron paramagnetic resonance (EPR) spectrum of the nitroxide. Both of these methods are at their best in the study of relatively rapid motions—those with correlation times shorter than a few times 10^{-7} s.

Recently a new EPR technique has been developed (Hyde and Dalton, *Chem. Phys. Lett.*, **16**, 568; 1972; Hyde and Thomas, *Ann. N.Y. Acad. Sci.*, **222**, 680;

1973) which promises to extend the application of the spin-labelling method into the time range 10^{-7}–10^{-3} s. This technique, known as saturation transfer EPR spectroscopy, depends upon the use of partially saturating microwave fields and out-of-phase detection to obtain a spectrum whose shape depends critically on the rate of rotational diffusion. The theory of the method is discussed by Thomas and McConnell (*Chem. Phys. Lett.*, **25**, 470; 1974); the maximum sensitivity to motion occurs when $\tau_c \sim 1/\omega_m \sim T_1$ ($\sim 10^{-5}$ s), where ω_m is the modulation frequency and T_1 the electron spin-lattice relaxation time.

This technique has now been applied to myosin and its interaction with actin (Thomas, Seidel, Hyde and Gergely, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 1729, 1975). The spin label was attached to a thiol in the globular 'head' (subfragment 1) of the myosin molecule. The correlation time of the isolated subfragment 1 was 1.8×10^{-7} s, and for myosin this increased to 3×10^{-7} s. Thus there is only about a 40% decrease in τ_c when subfragment 1 is cleaved from myosin, although the molecular weight decreases by a factor of more than four. There must therefore be considerable internal flexibility in the myosin molecule; the same conclusion was drawn earlier from closely analogous fluorescence experiments by Mendelson *et al.* (*Biochemistry*, **12**, 2250, 1973). The saturation transfer EPR technique can also be applied to supramolecular complexes of myosin, which are essentially beyond the range of previous methods. Aggregation of myosin into filaments led to a ten-fold increase in correlation time, indicating that there was still considerable flexibility of the 'heads' of the myosin molecules. In contrast, addition of actin to either subfragment 1 or myosin led to a very substantial immobilisation of the label, giving a correlation time of 10^{-4} s. This indicates that the globular 'head' of myosin is essentially irrotationally bound to actin.

The saturation transfer EPR method shares two problems with the other labelling methods. Motion of the label relative to the protein to which it is attached will obviously lead to erroneously short values of τ_c for the protein. Though this does not seem to happen in the case of subfragment 1, it may limit the application of these methods to some systems. In addition, for a non-spherical protein, rotational motion cannot strictly be described by a single correlation time, and the value obtained will depend on the orientation of the label relative to the hydrodynamic axes of the protein. But notwithstanding the difficulties in precise quantitation, it is clear that this new method is a valuable addition to the armoury of the physical biochemist interested in supramolecular structure.

review article

The origin of nuclei and of eukaryotic cells

T. Cavalier-Smith*

A new theory not involving symbiosis is proposed for the origin of eukaryotic cells. It explains how the evolution of phagocytosis by a wall-free blue-green alga would have created selection pressures leading directly to the formation of all characteristic eukaryote organelles and cell properties including mitosis, meiosis and sex.

THE problem of the evolutionary origin of eukaryotes has been a major one in biology ever since the fundamental distinction between prokaryotic and eukaryotic cells[1-3] became clear. It is generally accepted that eukaryotes evolved from prokaryotes, but how this happened is unknown[4-5]. Certain similarities between mitochondria and bacteria and between plastids and blue-green algae have led to the recent revival[6-8] of old theories[7-11] suggesting that these eukaryote organelles are derived from intracellular symbiotic prokaryotes and also to the suggestion[7,12,13] that microtubules, centrioles and flagella are similarly derived. This "serial endosymbiosis theory of the origin of eukaryotes", which supposes that eukaryotes evolved as a result of the symbiosis of from three to six[7,14-16] genetically different prokaryotes, has received more support[2-19] than the alternative theory that they evolved from a single prokaryote species by intracellular differentiation[20-22].

My strongest criticism of the symbiosis theory is that it fails to explain how the eukaryote condition itself (that is, the nucleus) evolved[24] Most proponents of the symbiosis theory[5-19] do not seriously discuss the origin of the nucleus, but assume it to have evolved gradually from a prokaryote nucleoid

Eukaryote nuclei differ in at least three fundamental ways from prokaryote nucleoids. They are surrounded by a double-membraned envelope bearing characteristic pores; they contain several non-identical chromosomes which are linear and not circular; segregation occurs by mitosis which always involves spindle microtubules. Here I show how these differences could have arisen and argue that the same selective forces would also have led to the formation of mitochondria, plastids and other characteristic eukaryote organelles and properties.

Like Stanier[5] I consider the evolution of endocytosis (phagocytosis and pinocytosis) to be of key importance in eukaryote evolution. But this is not because it enabled them to harbour endosymbionts. (I believe endosymbiosis to be one of many secondary and almost inevitable consequences of phagocytosis, but not the cause of the eukaryote condition.) It is because phagocytosis provided not only the selective pressure but also the physical mechanism (membrane budding and fusion) for cell compartmentation by intracellular membranes. Cell compartmentation explains not only the origins of mitochondria, plastids and nuclei, but also their characteristic properties more simply than does the symbiosis theory.

I assume that the ancestor of all eukaryotes was a single-celled, facultatively phototrophic, blue-green alga, unable to fix nitrogen but possessing oxygen-evolving photosynthesis and oxygen-using respiration based on cytochromes and other electron transport molecules borne on intracellular thylakoid

*Address: Department of Biophysics, University of London King's College, 26-29 Drury Lane, London WC2B 5RL, UK

membranes (Fig. 1a). The first step leading to eukaryotes must have been the loss of the cell wall by such an alga living in a shallow bacteria- and detritus-rich benthic environment (Fig. 1b). Cell wall degrading enzymes like those abundantly secreted by soil myxobacteria[3] may initially have produced a blue-green algal "L-form" which subsequently became a stable L-form. Whatever the mechanism, wall loss was essential for phagocytosis and explains the complete absence of peptidoglycan cell walls in eukaryotes.

Many blue-green algae (in contrast to bacteria, whose minute size would make the evolution of phagocytosis difficult) have cells as large as those of unicellular eukaryote algae[3,25], so size would be no barrier to the acquisition of phagocytosis—they would already be large enough to engulf bacteria. A strong selective force would favour any blue-green algal L-form able to develop phagocytosis and so become a "pre-alga". A phagocytic pre-alga could photosynthesise by day or during the Arctic summer, and phagocytose by night or during the Arctic winter (or in any dark environment). This versatility would give it a clear advantage over other blue-green algae (mostly obligate phototrophs) as well as over bacteria since it would seldom lack food.

Evolution of phagocytosis

I suggest that there is a fundamental similarity between the mechanism of plasma membrane budding to form a phagosome and the mechanism of eukaryote cell cleavage during cytokinesis. Both involve invagination, breakage and resealing of membranes. Clearly they differ in the timing and location of these processes. But I think these reflect differences in control rather than in the basic mechanism, and that the blue-green alga evolved cleavage first and endocytosis evolved subsequently from it.

Phagocytosis, like division, reduces the surface area of the plasma membrane but, unlike division, creates separate intracellular phagosome membranes. Extensive endocytosis is therefore not possible unless the phagosome membrane can refuse with the plasma membrane after absorption of its contents. Thus phagocytosis could not evolve in the absence of this reverse process (exocytosis—frequently the basis of secretion in eukaryotes). Since, in growing (but not in non-growing) cells, limited exocytosis is possible in the absence of endocytosis, exocytosis probably evolved before endocytosis (either as a mechanism of membrane growth additional to the insertion of individual molecules into an existing membrane, or more probably for secretion of extracellular digestive enzymes). Any blue-green alga possessing both cleavage and exocytosis would be preadapted for the evolution of phagocytosis. Figure 1 shows a possible evolutionary sequence.
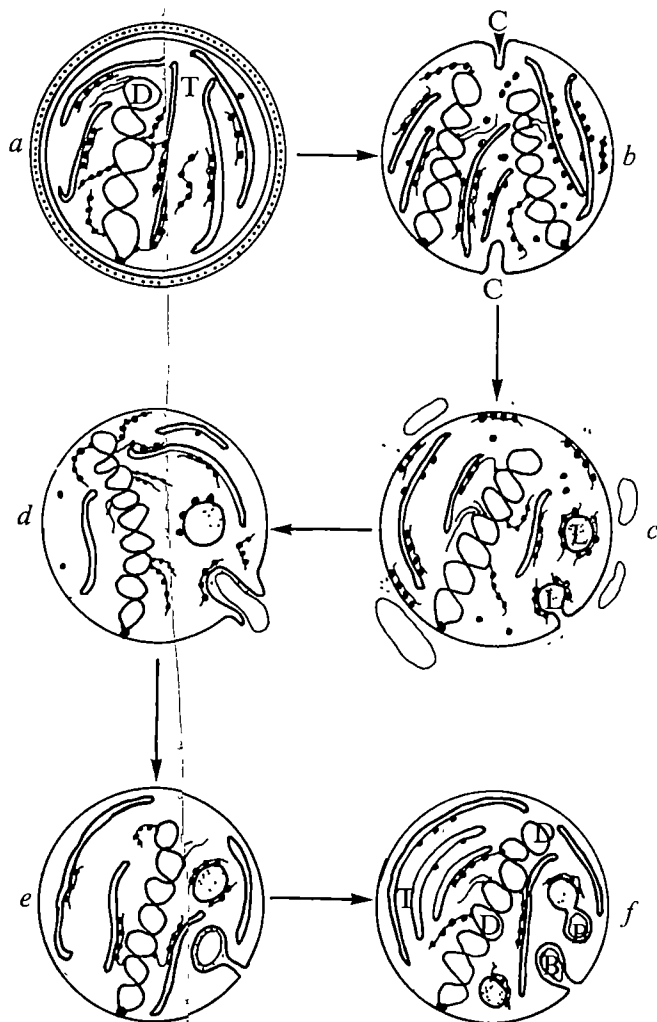
Fig. 1 The evolution of phagocytosis A blue-green alga loses its cell wal (*a*) and evolves an acto-myosin dependent cell cleavage mechanism (*b*). The resulting L-form develops extracellular digestion (*c*) by direct secretion of digestive enzymes across the plasma membrane and/or by exocytosis by protolysosomes (L) derived from thylakoids specialising in the intracellular storage of digestive enzymes. Efficiency is increased by partially (*d*) or completely (*e*) surrounding the prey before liberating the enzymes. Finally in the fully phagocytic "pre-alga" only the plasma membrane engulfs the bacterium (B) and the protolysosome fuses with the resulting phagosome. DNA (D), with its attached polysomes, and most thylakoids (T) remain unchanged.

I suggest that not only exocytosis, endocytosis and cleavage but all cases of controlled membrane budding and fusion in eukaryotes (for example, budding of smoth vesicles from rough endoplasmic reticulum or Golgi apparatus, or the fusion of transmitter vesicles with presynaptic membranes) have a common basic mechanism, which I call cytosis. Since much of eukaryote cell evolution can be understood in terms of a diversification in the uses of and increased control over the timing and positioning of cytosis it is a pity that so little is known of its mechanism. Conceivably membranes containing polyunsaturated fatty acids (found in blue-green algae and in eukaryotes but not in bacteria[3]) were a prerequisite. Although phospholipid membranes have a natural tendency to bud and fuse I suggest that cytosis also universally involves a calcium-activated contractile actin–myosin-like microfilament system[26]. The smplest explanation for the universality of actomyosin in eukaryotes[2] is that it was the essential molecular innovation which made the origin and evolution of eukaryotes possible, and that it originated when our blue-green algal ancestor lost its cell wall and the prokaryote type of cell division by located growth of a semi-rigid membrane was

superseded by actin–myosin mediated cleavage by furrowing, that is by cytosis.

Subsequently, modifications led to exocytosis and then phagocytosis. Then selection for greater phagocytic efficiency would occur. Cells would be selected for greater size and for the ability to phagocytose over their whole surface. The actomyosin system would diversify independently to produce amoeboid movement—greatly increasing predation efficiency—and cytoplasmic streaming and organelle movement—speeding up contact between phagosomes and protolysosomes.

Origin of the spindle

Such a highly mobile cell surface would interfere with chromosome segregation (dependent in prokaryotes on attachment to a stable semi-rigid membrane[2]). Endocytosis of the chromosome attachment site would be especially serious, so there would be strong selection for a new rigid non-membrane segregation mechanism—the microtubule. Initially microtubules joined the two membrane attachment sites (Fig. 2*a*) and pushed them apart as they grew (like pole-to-pole spindle microtubules, the only universal components of modern spindles[24,29]; or like the reverse of sex pilus retraction (microtubules and sex pili are both tubular and interact with chromosome attachment sites—are they related or just functionally similar?)). This ensured that one chromosome ended up on each side of the cleavage furrow (a mechanism to ensure that cleavage was at the equator was also essential).

Efficient segregation by microtubules ended the selective advantage of chromosome attachment to the plasma membrane; the attachment site would soon be endocytosed and thereafter remain inside the cell as a protonuclear envelope (Fig. 2*b*), making the entire cell surface available for phagocytosis. The origin of mitosis was not a consequence, as commonly supposed, of the greater size or greater number of eukaryote chromosomes but was instead the essential prerequisite for these changes.
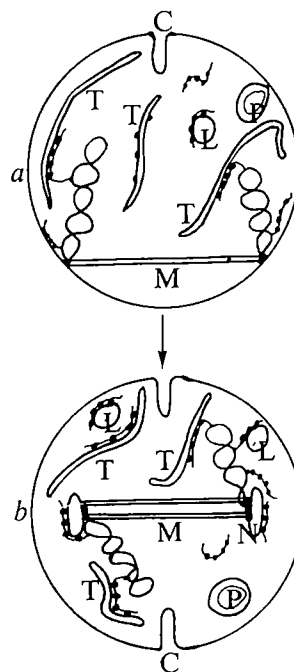
Fig. 2 The evolution of spindle microtubules (M) in the amoeboid pre-alga, as a device to push the two circular daughter chromosomes (here shown twisted into supercoils) apart to opposite poles of the cell to ensure that one is present in each daughter cell produced by the cleavage furrow (C). Initially chromosomes were attached to the cell membrane (*a*) but later (*b*) the attachment sites were endocytosed to become protonuclear envelope, N. Phagosomes (P), protolysosomes (L) and thylakoids (T) are also shown
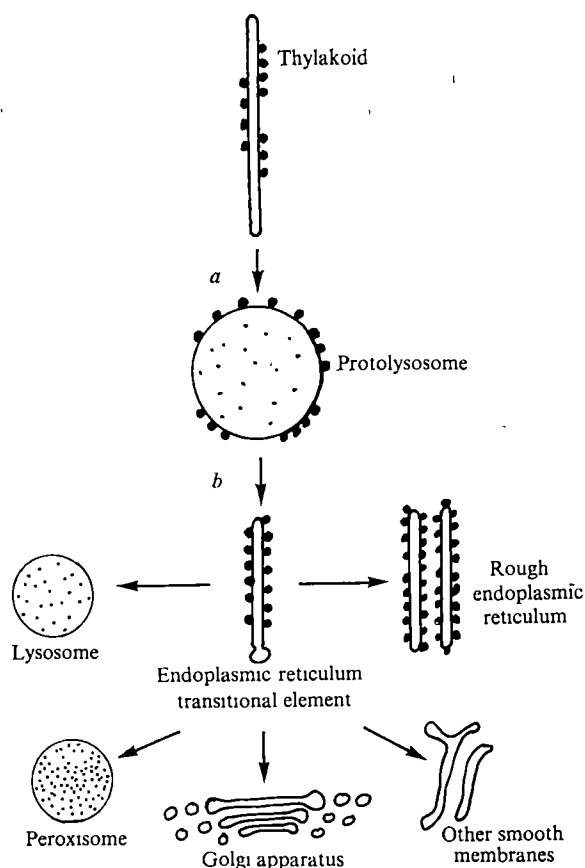
Fig. 3 Diversification of the (ribosome-covered) protolysosome (a) (originally derived from a ribosome-bearing thylakoid). The evolution of membrane budding by cytosis allowed differentiation and specialisation between the "rough" ribosome-bearing membranes and their various smooth-membraned products (b)

Cytosis and origin of sex

Cytosis also provided the mechanism for cell fusion, which must have evolved in a wall-free cell, and initially was probably poorly controlled and more frequent than today. Its initial selective advantage would be greatest in times of starvation (starvation still triggers sexual differentiation in many algae and fungi) when fused cells would have twice the internal food supply of unfused competitors. I suggest that it originated in this way in the amoeboid pre-alga before even the development of the nuclear envelope; many properties of eukaryote chromosomes are best explained by the simultaneous evolution of mitosis and meiosis.

With only one circular chromosome per cell meiosis was unnecessary; the primitive mitosis described above would ensure segregation Recombination was easy in the absence of a nuclear envelope. Assuming reciprocal and random recombination (as in the *rec* system of *Escherichia coli*), an even number of crossovers would produce two recombined daughters but an odd number would produce double length circular DNA. This increase in genome size and redundancy would immediately provide raw material for rapid evolution of new functions and create multiple replicon origins. The random release of spare copies of genes from stabilising selection pressure would break up operons. But an indefinite increase in DNA would be disadvantageous (and double-sized DNA molecules would often be broken during segregation because of their two membrane-microtubule attachment sites), and so selected against—most simply by the chromosomes becoming linear by the mechanism I previously suggested[30]. With linear chromosomes odd as well as even numbers of crossovers will give normal reciprocal recombinants thus terminating the explosive burst of new genome creation.

Selection for increased recombination efficiency would lead to chromosome pairing mediated by a synaptinemal complex. Efficient pairing would remove the necessity for having all the DNA in one molecule (previously necessary to prevent aneuploidy). Indeed, positive selection for chromosome fragmentation by the mechanism previously suggested[30] is likely because this will give increased recombination by independent assortment of non-homologous chromosomes. Thus the most distinctive features of eukaryote chromosomes, mitosis, meiosis and sex probably all evolved in a very short space of time, during the earliest stages of eukaryote evolution as a direct consequence of the evolution of cytosis. Chloroplast fusion[31] was probably a late development.
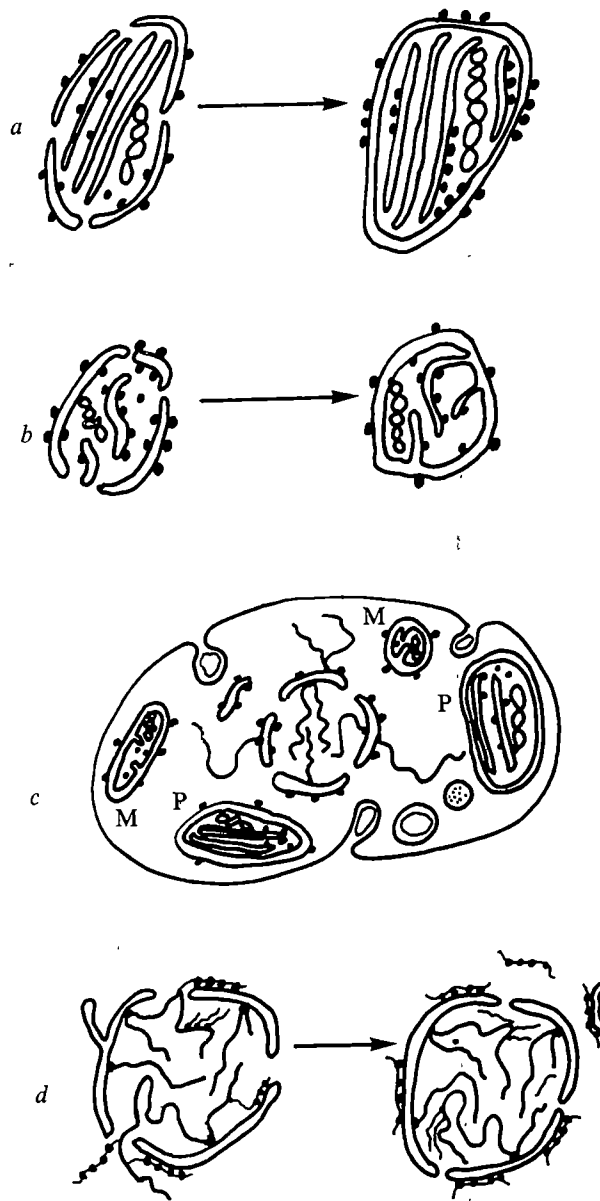
Fig. 4 The evolution of plastids, mitochondria and nuclei by fusion of DNA-associated thylakoids or endoplasmic reticulum cisternae. a, Plastids resulted from association of photosynthetic and plasmid-associated thylakoids to form a compartment containing DNA, ribosomes and Calvin cycle enzymes. b, Mitochondria resulted from fusion of plasmid-associated respiratory thylakoids to form a compartment containing Krebs cycle and fatty acid metabolising enzymes. c, Cell at intermediate stage with three distinct compartments: mitochondria (M), plastids (P) and nucleocytoplasm, whose protonuclear envelope (N) with six linear chromosomes will eventually fuse (d) to form a nuclear envelope separating the cytoplasm with protein synthesising enzymes from the nucleus with DNA and RNA synthesising and processing enzymes.

Cell compartmentation and organelle origins

Increase in cell size and in diversity of cell components in response to the selection pressures described above would dilute each component and lower the efficiency of reactions. This could be prevented only by vastly increasing the amounts made and the total concentration of materials or by dividing the cell into compartments each specialising in different functions. Cells adopting the latter course would have a tremendous selective advantage. The evolution of cytosis not only provides the selective pressure but also the mechanism

Fig. 5 A possible pattern of eukaryote diversification following the formation of the first true eukaryote (red algal-like) "proto-alga"
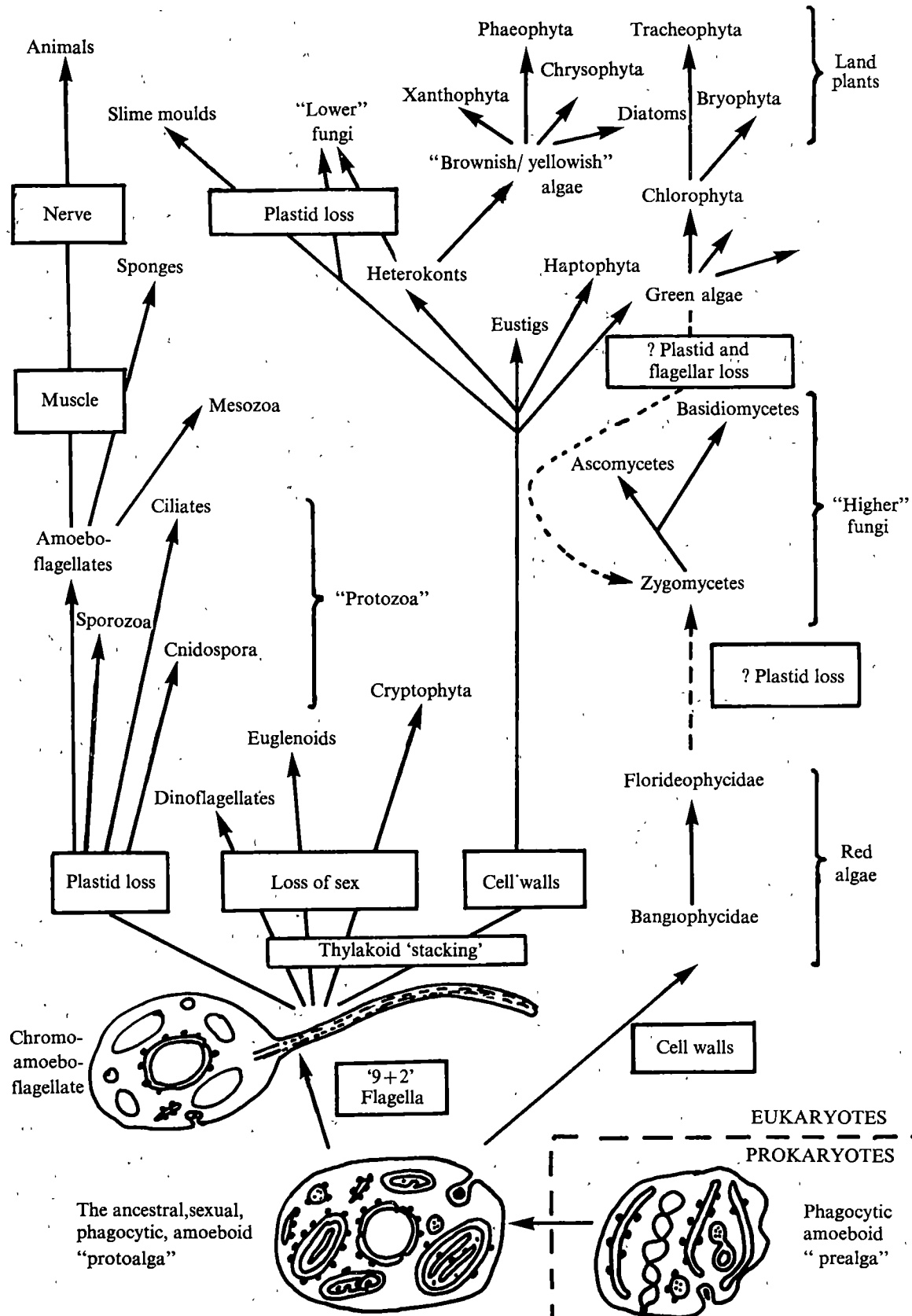
for compartmentation.

The early ribosome-covered protolysosomes (Fig. 1) were already separate compartments (because of the need to secrete, and protect the cell from, their hydrolases) and could very simply have differentiated into rough and smooth endoplasmic reticulum, lysosomes, peroxisomes and Golgi apparatus as shown in Fig. 3; they and the original thylakoids could have formed the plastid, mitochondrial and nuclear envelopes by fusion as shown in Fig. 4.

Initially ribosomes, DNA, RNA and nucleic acid polymerases were divided into 3 compartments, Fig. 4c. The nucleoid in the cytoplasmic compartment was segregated by microtubules and its DNA became linear and fragmented as suggested above. Chloroplast and mitochondrial plasmids were present always in large enough numbers per cell not to require a special segregation mechanism, and so (generally) remained circular because with multiple copies the selection pressure for linearity was much less. In both plastids and mitochondria, DNA, messenger RNA and ribosomes are in the same compartment (as in bacteria) so would be expected to retain many prokaryotic properties. By contrast nuclear DNA and RNA became segregated from cytoplasmic RNA and ribosomes (Fig. 4d) so ribosomes could no longer start translating messenger while it was still being made and new mechanisms were required for the transport of messenger and nascent ribosomal subunits across the nuclear envelope. (Nuclear pores allowing free passage of small and medium sized molecules, but not larger ones and macromolecular complexes[32], probably arose in response to the need to regulate nucleocytoplasmic exchange.) Nucleocytoplasmic separation would have imposed new selective forces on DNA and RNA. Though it is premature to speculate on their nature I predict that they will go far to explain other differences between nucleocytoplasmic genetic systems on the one hand and prokaryote/mitochondrial/plastid ones on the other.

The origin of the unique features of cytoplasmic ribosomes and of nucleoli probably dates from the time when the pre-alga became completely compartmented to form the first true eukaryote—the proto-alga (Fig. 5). I suggest that originally the pre-algal plasmids and nucleoid had identical ribosomal DNA present in multiple tandem copies (many copies were needed because of the large cell size), and that this identity was maintained by recombination involving a ribosomal DNA episome (replicating as a rolling circle[33]). But, after complete compartmentation, DNA and ribosomes could no longer cross the membranes (but proteins could be secreted directly across them by membrane-bound ribosomes[34]), so plasmid and nuclear ribosomal DNA then evolved independently. This predicts that gene amplification and rolling circle replication of nucleolar DNA will be found universally in eukaryotes. The existence of a distinct nucleolus is connected with the need to transport both ribosomal and messenger RNA across the envelope.

Nucleocytoplasmic separation, the breakup of operon-like gene clusters and the production of large amounts of redundant DNA as suggested above, were probably enough to lead to completely novel systems of gene regulation in eukaryotes[36] which would subsequently make possible the evolution of highly differentiated multicellular organisms. In future research it will be important to determine, by careful comparison of primitive unicellular eukaryotes and differentiated multicellular ones, which features (for example, giant heterogeneous nuclear RNA[35], repetitious DNA[36], palindromes[37]) are universal features of eukaryote genetic systems and which are specifically associated with complex multicellular differentiation. ·

Comparison with symbiosis theories

My model is superior to symbiosis theories in three main ways. First, it explains how eukaryote nuclei evolved. In doing so it provides a plausible selective advantage for the evolution

of all major features of eukaryote nuclear structure and genetic systems (except the presence of histones). According to my theory the absence of sex in an entire major eukaryote group, for example euglenoids[38], is the result of secondary loss and not a primitive feature. The absence of histones in dinoflagellates[39] might also be a secondary feature perhaps resulting from the loss of sex. If so the primary function of histones could have been efficient packing of DNA to facilitate meiotic pairing and segregation.

Second, it gives plausible reasons for the differences between nucleocytoplasmic and prokaryote/plastid/mitochondrial genetic systems. This the symbiotic theories do not do; they ignore the fact that both kinds of genetic systems must have evolved from prokaryote systems, and so resemblances are not at all surprising[21]. The real problem is why the nucleus is different, which symbiosis does not explain.

Third, it is far simpler than any symbiotic theory. These postulate two to five separate symbiotic events[16]. Though the symbiotic origin of mitochondria and chloroplasts is a possibility (and one compatible with the origin of nuclei by the mechanism proposed here), the resemblances between them and prokaryotes are to be expected on either theory. The symbiotic origin of flagella and the mitotic apparatus[7,12], is untenable for many reasons[8,24]. The idea that the complexities of sex and meiosis evolved independently 27 times (Fig. 2–6 of ref. 7) in much the same way is incredible.

Eukaryote diversification

I regard the absence of flagella in red algae (possibly also in higher fungi if they evolved from them by plastid loss[40]) as primary[20,21,24]; they are probably the most primitive living eukaryotes. Though some amoebae without flagella could have evolved from primitive red algae by plastid loss, most eukaryotes must be derived from a red algal line which evolved 9+2 flagella from aggregates of microtubules. The selective advantage is obvious: they could colonise a completely empty niche as the first marine phytoplankton (or if, unlike today[25], there were then blue-green algae in the open sea, become their first predators) through their new ability to stay in suspension and migrate to the level most suitable for photosynthesis and (or) predation. By loss of phycobilins and the development of other pigments a great variety of brownish and green phytoflagellates were formed[39,41,42]. These were the ancestors of all plants and, by plastid loss, of non-photosynthetic protists and animals (Fig. 5). The generality of the circadian clock in eukaryotes (and possible absence in bacteria) is easiest to understand if it evolved in a photosynthetic common ancestor to maximise photosynthesis by day and division by night. Complex polysaccharide–protein cell walls or surface coats, often secreted by the Golgi apparatus, evolved in many lines. Adhesion between such extracellular layers led to multicellularity, in many separate lines[43,44].

Comparative studies clearly indicate that centrioles are derived from basal bodies[45] and probably became secondarily associated (perhaps on several independent occasions) with the spindle poles, and are not essential for mitosis[24,46]. Chromosome-to-pole spindle microtubules probably evolved long after the origin of mitosis, but only in some eukaryote groups[24]. Microtubules have also independently evolved into many other organelles of motility, such as axostyles[47], axopods[48], suctorian tentacles[49] and haptonemata[30] as well as numerous structural elements as in the cortex of protist cells[39,50], the phycoplast[51] or neurotubules[52].

My view that the origin of all eukaryotic organelles and genetic systems can be traced back to two fundamental innovations, (1) cytosis involving actomyosin and (2) microtubules, has many implications (too numerous to discuss properly here) which can be tested by observations on living organisms. Unfortunately evidence from the fossil record[42,53] will be very hard to come by because the ancestral eukaryotes had no cell walls and because the changes postulated could have occurred very rapidly indeed, possibly in a very restricted locality.

1 Stanier, R. Y., and van Niel, C B., *Archs Mikrobiol*, **42**, 17–35 (1962).
2 Stanier, R. Y., Doudoroff, M. and Adelberg, E. A., *General Microbiology* (third ed.) (Macmillan, London, 1971)
3 Brock, T. D, *Biology of Microorganisms* (Prentice-Hall, Englewood Cliffs, New Jersey, 1974).
4 Echlin, P., *Symp. Soc gen. Microbiol.*, **20**, 221–248 (1970)
5 Stanier, R. Y., *Symp. Soc. gen Microbiol*, **20**, 1–38 (1970)
6 Ris, H., and Plaut, W., *J. Cell Biol.*, **12**, 383–391 (1972)
7 Margulis, L., *Origin of Eukaryotic Cells* (Yale University Press, New Haven, Connecticut, 1970)
8 Taylor, F. J. R., *Taxon*, **23**, 229–258 (1974)
9 Schimper, A. F. W , *Bot. Z.*, **41**, 105–114 (1883)
10 Altman, R., *Die Elementar Organismen und ihre Beziehung zu den Zellen* (Veit, Leipzig, 1890)
11 Mereschkowsky, C., *Bull. Soc Sci. Nat Ouest France*, **6**, 17–21 (1920).
12 Sagan, L., *J theor Biol.*, **14**, 225–274 (1967)
13 Margulis, L., *Bio Systems*, **6**, 16–36 (1974)
14 Raven, P. H., *Science*, **169**, 641–646 (1970).
15 Lee, R E , *Nature*, **237**, 44–46 (1972)
16 Margulis, L., *Evol. Biol* , **7**, 45–78 (1974)
17 John, P , and Whatley, F R , *Nature*, **254**, 495–498 (1975)
18 De Duve, C., *Science*, **182**, 85 (1973).
19 Hall, J. B., *J. theoret. Biol.*, **38**, 413–418 (1973).
20 Klein, R., and Cronquist, A , *Q Rev Biol.*, **42**, 105–296 (1967).
21 Allsopp, A , *New Phytol.*, **68**, 591–612 (1969).
22 Raff, R. A., and Mahler, H. R., *Science*, **177**, 575–582 (1972)
23 Bell, P. R., *Symp. Soc exp. Biol.*, **24**, 109–128 (1970)
24 Pickett-Heaps, J. D , *Biosystems*, **6**, 37–48 (1974).
25 Fogg, G E , Stewart, W. D. P., Fay, P , and Walsby, A. E., *The Blue Green Algae* (Academic, London and New York, 1973).
26 Axline, S G., and Reaven, E P , *J. Cell Biol.*, **62**, 647–659 (1974)

27 Pollard, T. D , and Weihing, R R., *Critical Reviews in Biochemistry*, **2**, 1–65 (CRC, Cleveland, Ohio, 1975)
28 Higgins, M. L., and Schockman, G. D , *Crit Rev. Microbiol*, **1**, 29–72 (1971)
29 Oakley, B. R , and Dodge, J D., *J. Cell Biol.*, **63**, 322–5 (1974)
30 Cavalier-Smith, T , *Nature*, **250**, 467–470 (1974)
31 Cavalier-Smith, T., *Nature*, **228**, 333–335 (1970).
32 Paine, P L , Moore, L C , and Horowitz, S. B., *Nature*, **254**, 109–114 (1975)
33 Hourcade, D., Dressler, D., and Wolfson, J , *Cold Spring Harb. Symp quant. Biol.*, **38**, 537–550 (1974).
34 Kellems, R E , Allison, V. F , and Butow, R. A , *J Cell Biol.*, **65**, 1–14 (1975)
35 Britten, R J , and Davidson, E. H , *Science*, **165**, 349–357 (1969).
36 Davidson, E H., and Britten, R. J., *Q Rev Biol* , **48**, 565–673 (1973).
37 Wilson, D. A , and Thomas, C A., *J molec. Biol* , **84**, 115–144 (1974)
38 Leedale, G , *Euglenoid Flagellates* (Prentice-Hall, Englewood Cliffs, New Jersey, 1967).
39 Dodge, J D , *The fine structure of algal cells* (Academic, London, 1973)
40 Fritsch, F. E , *The Structure and Reproduction of the Algae*, **2** (Cambridge University Press, London, 1945).
41 Leedale, G., *Taxon*, **23**, 37–47 (1974)
42 Loeblich, A. R., *Taxon*, **23**, 277–290 (1974).
43 Picken, L., *The Organisation of Cells* (Oxford University Press, London, 1960).
44 Bonner, J. T , *The Evolution of Development* (Cambridge University Press, London, 1958)
45 Fulton, C , in *Origin and Continuity of Cell Organelles* (edit. by Reinert, J., and Ursprung, H ,) 170–221 (Springer, Berlin, 1971)
46 Cavalier-Smith, T., *J. Cell Sci.*, **16**, 529–556 (1974)
47 Bloodgood, R. A., and Miller, K. R , *J Cell Biol* , **62**, 660–671 (1974).
48 Ockleford, C D , and Tucker, J B , *J Ultrastruct. Res* , **44**, 369–387 (1973).
49 Rudzinska, M. A., *J Cell Biol.*, **25**, 459–477 (1965).
50 Huang, B., and Pitelka, D. R , *J. Cell Biol.*, **57**, 704–728 (1973).
51 Pickett-Heaps, J. D , and Marchant, H J., *Cytobios.*, **6**, 255–262 (1972).
52 Burton, P R., and Fernandel, H. L , *J Cell Sci* , **12**, 567–583 (1973)
53 Schopf, J. W , *Evol Biol* , **7**, 1–43 (1974)

articles

Palaeolithic remains at the Hadar in the Afar region

Gudrun Corvinus

Institut für Urgeschichte, University of Tübingen, Schloss, 74 Tübingen, Germany

Studies of Plio–Pleistocene deposits along the Awash River in the Hadar region of Ethiopia have revealed for the first time several Palaeolithic sites. In addition to a large number of artefacts which provide evidence of early to late Stone Age industries the deposits have also yielded a rich vertebrate fauna including what may be the earliest hominid remains yet discovered.

NEAR the confluence of the rivers Awash and Hadar, about 60 km ESE of Bati, the latter river has cut deeply into the gravel covered surface of the floor of the Ethiopian Rift (see Fig. 1) and has exposed 105 m of fluviatile, lacustrine and tuffacious sediments of Plio–Pleistocene age.

The flat surface of the rift floor consists of an extensive boulder gravel, 3–6 m thick, which lies unconformably on the Upper Pliocene and Lower Pleistocene sediments of the Hadar Series. This gravel extends from the foot of the Ethiopian Rift escarpment in the west, thinning slowly to beyond the Awash River in the east[1]. It seems to have been deposited as a series of large confluent fans derived from the escarpment, which form an extensive behada or peripediment. It is not cemented and consists of well rounded to subrounded boulders and pebbles 30–40 cm in size, set in a matrix of sandy, calcareous silt. The pebbles are neither sorted nor oriented into any stream direction. The gravel is probably of Middle Pleistocene age, as indicated by Early Stone Age (ESA) implements found *in situ*; no fauna has yet been found.

Beneath the boulder gravel the Hadar has exposed a 105-m section of the Hadar sediments—Upper Pliocene and Lower Pleistocene fossiliferous beds of sandstones, sands and clays, and occasional tuffs. A very rich vertebrate fauna indicates a relative age of more than 3 Myr for these beds[2].

Fossils of *Australopithecus*, at least 3 Myr old[3], were found for the first time during the survey of the Lower Hadar sediments by members of the 1973 International Afar Expedition. These included three fragments of femur and one fragment of tibia from a small hominid and a piece of cranium (mastoid) from a more robust *Australopithecus*.

In the Hadar area the terraces of the Awash River are not very pronounced. The Awash meanders in a flood plain about 1 km wide. In the inner parts of the meanders the silty and fine-sandy, modern alluvial plain is covered by a thick riverine forest comprising various species of acacia and tamarisk. The height of the plain is not more than 2 m above low water level.

A terrace 3–5 m high runs along the inner meanders beyond the riverine forest. It is covered by a thin, bouldery gravel within a sandy matrix. The same terrace also stretches along the Denen Dora tributary further west. There, a broad terrace covered by large boulders and pebbles extends between the Denen Dora and the Sidi Hakoma tributary, rising to 4 m above the bed of the Denen Dora.

Any pre-existing higher terraces of the Awash and the Hadar have been destroyed by erosion. Significantly, many—though not all—of the Badland Hills, 25–90 m in height along the Awash, have a covering of loose, well-rounded boulders in a sandy–silty matrix lying unconformably on earlier Hadar deposits.

All the boulders which cover the river terraces and erosional surfaces in the Hadar area have been derived from the erosion of the Middle Pleistocene boulder gravel. The pebbles of this
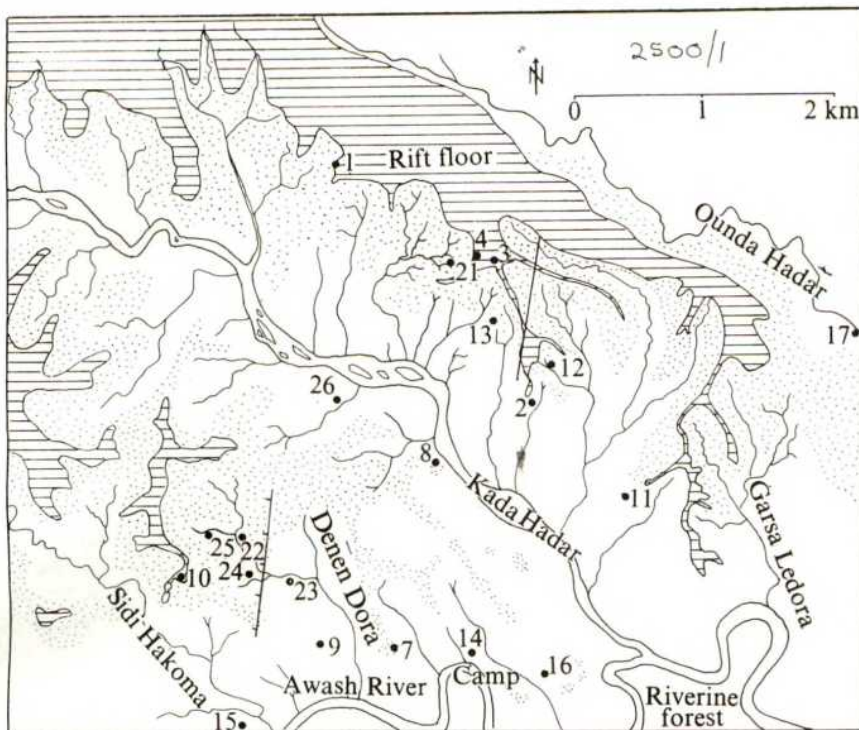
Fig. 1 Sketch map of the Hadar region. Stippled areas, Badland Hills; horizontal lines, rift floor more than 100 m above the Awash River; long straight lines, faults; ●, artefact localities.

boulder gravel consist of amygdaloidal basalt, trachyte, rhyolite and ignimbrite, derived from the Tertiary Trapps of the Ethiopian Plateau. They have a red-brown to dark-brown polish when they have been exposed for a long time. The boulders on the erosional surfaces and terraces are of the same type but they often have a deeper, sometimes black polish.

We may assume that the boulder cover of the Badland Hills represents a residue of boulder spreads which were redeposited on erosional surfaces during the downcutting of the area and which were originally derived from the boulder gravel of the Ethiopian Rift floor.

Artefact sites

Artefacts of all ages occur everywhere in the region (Fig. 1) both on the surface and in the river gravels. At some localities up to 45 specimens were found. They occurred mostly on the surface and were rarely *in situ*, except for a few Middle Acheulian bifaces found in recent, reworked river gravels. In most cases the artefact assemblages were mixed; that is, Middle Stone Age (MSA) flakes and cores have been found together with Late Stone Age (LSA) flakes, cores and waste. Choppers and bifaces also occur occasionally among them. The bifaces, choppers, modified pebbles, and the MSA flakes and cores, are all made from the local boulders of trachyte and basalt. The LSA people however, used siliceous material, such as jasper, chalcedony and, rarely, fine grained basalt. The subrecent specimens are of obsidian and quartz.

A few sites, however, contained assemblages of an homogeneous character. Sites Hdr 9 and Hdr 22 (Fig. 1) are Acheulian sites situated on the low terrace of the Denen Dora tributary. Bifaces were found at site Hdr 9 on the surface of the wide, flat, 4-m terrace of the Denen Dora near the confluence with the Awash. At site Hdr 22 the assemblage was found on the 1.50–3.00-m recent terrace in the middle part of the stream. The bifaces from that site do not, on the whole, have a dark-brown polish but have a grey to white, calcareous patination or no patination at all. They must therefore have been eroded out from the bouldery deposit of the terrace, rather than having been brought down from a higher level; most of the other pebbles on the surface and hill slopes are polished, having been exposed for a long time. The majority have been rolled down from the surrounding hills but no artefacts were found amongst them there. It is therefore necessary to ascertain from where the tools are derived. The Acheulian bifaces are all quite fresh

and show no evidence of river transport. But a few artefacts which are quite rolled and of a rather primitive appearance represent evidence of an older industry elsewhere.

I surveyed the whole of the Denen Dora river up to the source at the escarpment of the Ethiopian Rift floor and noticed that no artefacts could be found upstream of site Hdr 22. This suggests that the tools from site Hdr 22 were not brought down from the boulder gravel covered rift floor by the Denen Dora

Fig. 2 Bifacial choppers from site Hdr 10-III: *a*, from surface; *b*, found *in situ*.
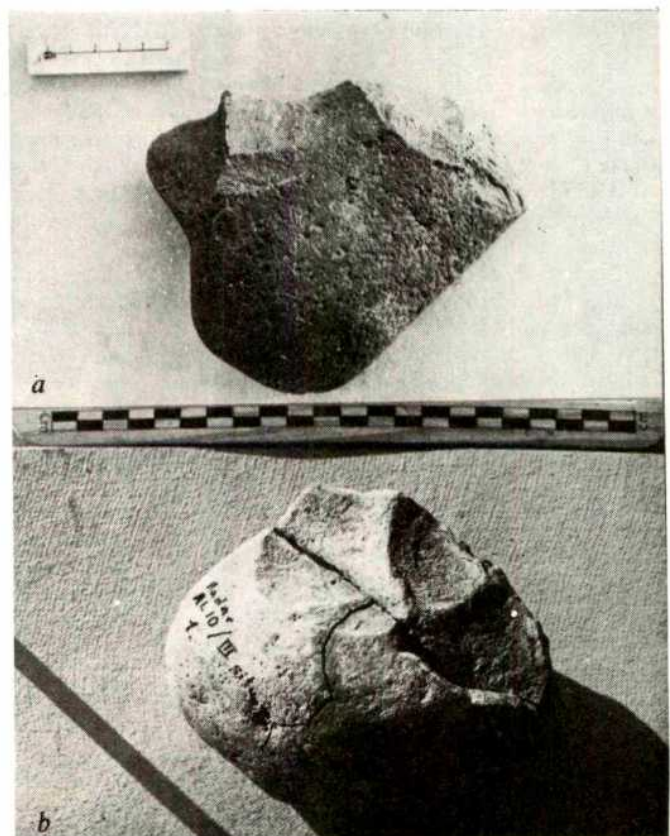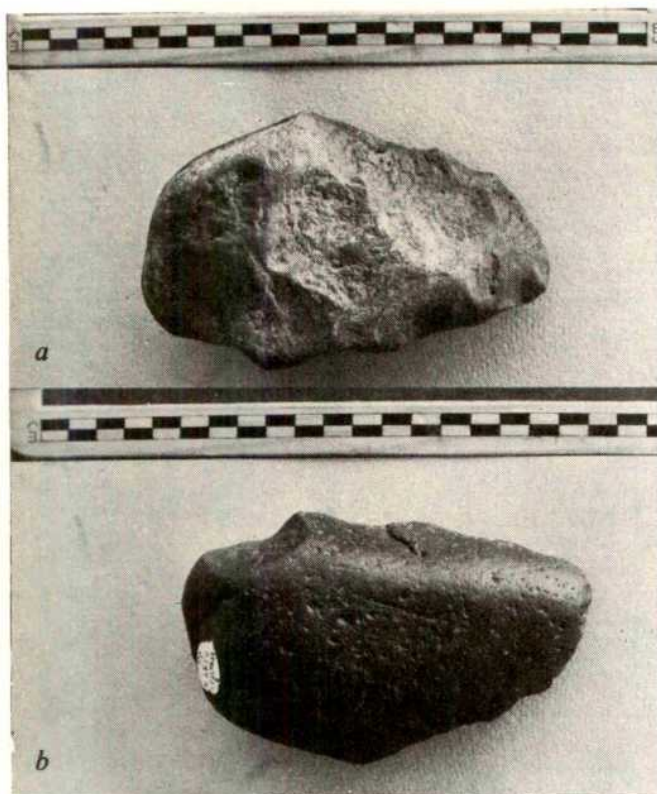
Fig. 3 Unifacial protohandaxe from site Hdr 22; *a*, ventral face; *b*, dorsal face, which is of cortex, showing the typical black polish.

River, but must derive from nearby, the original site having been destroyed.

A few very rolled, primitive bifaces and a chopper, however, were found in a small stream bed, which joins the Denen Dora from the west and which descends directly from the rift floor at site Hdr 10 (Fig. 1).

There is, therefore, evidence that at least two different types of industry were developed along the Denen Dora river. The first dates from the Middle Acheulian with rather fresh, well-made bifaces. The second, apparently older industry produced protohandaxes, choppers and crude flakes of rolled appearance.

Site Hdr 10 is situated immediately to the west of the Denen Dora on a narrow remnant of the otherwise eroded rift floor, where the boulder gravel is 3–4 m thick above the Hadar Series. At site Hdr 10-III, within an area of about 50 by 70 m, I found 32 second type artefacts on the surface and one within the boulder gravel.

These artefacts, lying among a large number of boulders and pebbles with a light-brown to dark-brown polish, seem to have come originally from within the boulder gravel. Most of them have a dark-brown polish, some of them only a light polish, and the *in situ* chopper has no polish at all but a thin crust of white lime like that on the pebbles within the boulder gravel. The artefacts comprise choppers, polyhedral and modified pebbles, crude flakes and cortex flakes and primitive handaxes or protohandaxes.

The few second type artefacts in the Denen Dora must have been brought down from the boulder gravel of site Hdr 10-III. At sites Hdr 10-I and II, I also found a few crude artefacts, some *in situ* at the edge of the boulder gravel; I dug two trial trenches into the gravel, one at each site to verify the origin. I excavated nine artefacts from the small trenches and I found eight *in situ* in the gravel along the edges. Forty-four artefacts were found on the surface.

Artefacts

Artefacts found in the Hadar area can be placed in five categories. First, at sites Hdr 10-I, II, III, (and occasionally from sites Hdr 22, Hdr 24 and Hdr 25) there were choppers, polyhedral and modified pebbles, crude flakes and protohandaxes; second, mainly at sites Hdr 22, Hdr 23, and Hdr 9, were found bifaces and a few flakes of the Middle Acheulian type (this category includes occasional pieces from other localities); third, mainly at sites Hdr Camp 14, and Hdr 11 (with occasional pieces from other localities) there were flakes and cores of a Middle Stone Age industry, mostly without retouch, struck off from prepared cores with prepared, thinned talons; fourth, mainly at sites Hdr 1, Hdr Camp 14, Hdr 15, Hdr 18 (and a few other places), there were flakes, cores and waste of a Late Stone Age industry, made of jasper, chalcedony and various other siliceous material; fifth, mainly at sites Hdr 3, Hdr 4, and Hdr 19 (and other localities), flakes, cores and waste from subrecent to recent industries, made of obsidian and quartz.

At site Hdr 10 the choppers, modified pebbles and protohandaxes are all made of Trapp material. The choppers usually have a bifacial edge, formed by alternate flaking in two directions, though sometimes they are unifacial, formed by having a few flakes struck off in one direction only; the rest is cortex (Figs 2 and 3). Interestingly, a number of choppers seem to be transitional to bifaces or unifacial handaxes. These tools display unifacial or alternate bifacial flaking at one end of the pebble in such a way that a rounded or irregular point, or a point and part of a lateral edge is produced.

Some crudely worked pieces have been classified as modified pebbles. Polyhedrons and discoids are rare. Spheroids are absent.

The protohandaxes are a special group of artefacts showing much individual variation. Ten specimens were collected from sites Hdr 10, three from the tributaries of the Denen Dora and ten from sites Hdr 22 and Hdr 9 (Fig. 3). They show a few irregular flake scars. Much cortex remains not only at the butt end, but often over large parts of the tool. The edges are not regular; and often only one edge or part of one edge is produced with an asymmetrical, often blunt, point. In rare cases there are two edges, which are irregular, zig-zag, and with no secondary trimming. Some protohandaxes may have one face made entirely of cortex, so not all of these artefacts can be called protobifaces and the term protohandaxes is preferable. There are a few small triangular forms in which only the point has been worked bifacially.

The flakes have all been struck from unprepared, large cores (Fig. 4). The talons are plain with a large angle towards the flake surface, and the bulbs are pronounced. The dorsal face often consists partly of cortex, with only one or two former flake scars. There is no preferred shape. They may have been used, though the edges are usually irregular and often blunt.

The Acheulian artefacts from sites Hdr 22 and Hdr 9 were found mostly on the surface of the low terrace of the Denen Dora, though some were found *in situ*, redeposited in the terrace gravel.

On the whole, the bifaces are well-made, trimmed by large,

Fig. 4 Dorsal faces of flakes from site Hdr 10.

shallow flaking, with more or less biconvex sections and elongate-ovate shapes. The edges run around the whole of the circumference and are rather straight with secondary trimming in the finished specimens. Characteristically, almost all of them retain a small cortical part at the butt or, rarely, on the upper face. A few prepared cores (with basal preparation) and flakes from prepared cores also belong in this assemblage.

There are, however, a number of artefacts which are much cruder in appearance and which do not belong to the Middle Acheulian. These include crude flakes of the Hdr 10 type and a few protohandaxes. As they are all found on the surface it is possible that they originated at site Hdr 10 and were transported from there by the small, steep western tributary of the Denen Dora.

The Middle Stone Age flakes occur only on the surface in the Hadar area, not only on the lower terraces but at all levels of the eroded landscape around the Hadar River and on the floor of the Rift, too. It is, therefore, impossible to say anything about their stratigraphic context, or their age. Unlike the younger flakes, however, they are all made of basalt and trachyte and they may well be products of an Acheulian light-duty industry.

These flakes have been struck from prepared cores. The dorsal faces show primary flake scars and in many cases the talon has also been prepared. Evidence of retouch is extremely rare and any that does occur is possibly a result of chipping or battering by natural agencies during transport rather than intentional retouch. Many of the flakes are quite rolled. The shapes are mostly irregular, often with blunt edges so that a great part of them seems to be waste. But there are a few parallel-sided and pointed flakes. Cores are not abundant. There are rare tortoise-shaped cores, with a base formed by one or two flake scars; cores with a bifacial edge, with flakes removed alternately along the edge; cores with two platforms at right angles; and cores with two parallel platforms.

My thanks go to the Centre National de Recherche Scientifique, the National Science Foundation, and Mr Taieb, leader of the International Afar Expedition of 1973.

1 Taieb, M., thesis, Univ. Paris (1974)
2 Taieb, M., Johanson, D C , Coppens, Y , and Kalb, M. J., C r. hebd. Séanc. Acad Sci , Paris, sér D, 275, 819–22 (1972)
3 Taieb, M., Johanson, D. C., Coppens, Y., Bonnefille, R,. and Kalb, M. J., ibid. 279, 735–38 (1974).

Integration of viral genomes

V. M. Zhdanov

The D. I. Ivanovsky Institute of Virology, Moscow, USSR

DNA transcripts of infectious RNA viruses were found to be integrated in the DNA of chronically-infected tissue cultures. DNA sequences homologous to RNA of measles virus were found in tissues affected with systemic lupus erythematosus. These data open up a new class of virus–cell interaction that may be a result of cooperation between infectious and oncogenic viruses.

Two types of interaction between the virus and the infected cell are characteristic of virus infections: autonomous replication of the virus in the cell and integration of the viral genome into the host cell chromosome.

Until now, integration has been found only with the temperate bacteriophages, oncogenic viruses and the related 'slow' viruses. We have shown, however, that nuclear DNA of avian, mammalian and human cells can integrate genomes of classic infectious viruses such as measles virus[1] and arbovirus[2], and that a similar process occurs in systemic lupus erythematosus, an autoimmune disease of man[3]. Here I present the results of a study of integration processes both in artificial tissue culture and in clinical samples.

To study the molecular mechanisms of the persistence of virus in chronically-infected tissue cultures three systems were used. Details of the systems, chick embryo fibroblasts infected with measles virus (CEF-MV), mouse L cells infected with Sindbis virus (ML-SV), and human Hep-2 cells infected with tick-borne encephalitis virus (Hep-2 – TBEV), are given elsewhere[4-12]. Briefly, the chronically-infected cell lines were obtained by inoculating the cultures with low doses of virus (0.01 TCD$_{50}$ per cell of measles virus and 0.01 plaque-forming units (PFU) per cell of the arboviruses) with subsequent reimplantation of the cultures every 4–6 d. The CEF-MV line has now undergone more than 120 passages; ML-SV, more than 80 passages; and Hep-2–TBEV more than 600 passages. All three cultures show no signs of cytopathic changes and the viruses are regularly detected in the cytoplasm by immunofluorescence tests with virus-

specific sera. Infectious titres of intracellular viruses are low (10^2–10^3 TCD$_{50}$ per ml for MV; 10^2–10^3 PFU ml^{-1} for SV; and 10^3–10^4 for TBEV) and those of extracellular viruses even lower (1–10 for MW; 0–1 for SV; and 10–10^2 for TBEV).

Biophysical studies showed that in all three systems subviral structures are predominant in contrast to acutely-infected cells which produce mature viruses (Fig. 1). The specificity of viral ribonucleoproteins was confirmed by titration of infectivity.

These data were obtained in experiments where actinomycin D was added for 3 h. When the antibiotic was added for 12 h, the synthesis of virus-specific structures was strongly inhibited in chronically-infected cells, in contrast to acutely-infected cells, where the synthesis of virus-specific structures was resistant to the action of the antibiotic (Fig. 2) Similar data were obtained in experiments with measles and Sindbis viruses.

As actinomycin D inhibits the transcription of cellular DNA into RNA, DNA sequences homologous to the viral RNAs were looked for in the chronically-infected cells. ^{3}H-labelled RNA was hybridised with excess DNA from chronically-infected and uninfected cells. DNA from chronically-infected cells was found to contain sequences homologous to RNA of the corresponding viruses whereas no such sequences could be found in non-infected cells (Fig 3). DNA preparations from chronically-infected cells were then used to treat appropriate sensitive cells to see whether virus could be isolated from these treated cells. TBEV was isolated from SPEV cells treated with DNA from Hep-2 – TBEV cells (Table 1), and SV from the brains of mice inoculated with DNA from ML-SV cells. In both cases DNase destroyed the infectivity of the DNA preparations whereas RNase had no effect.

As none of the three viruses under study possesses virion-associated or virus-induced reverse transcriptase, the transcription of virus RNA into double-stranded DNA must have been accomplished by latent oncornaviruses present in the cultures. The chickens used as a source of the CEF-MV
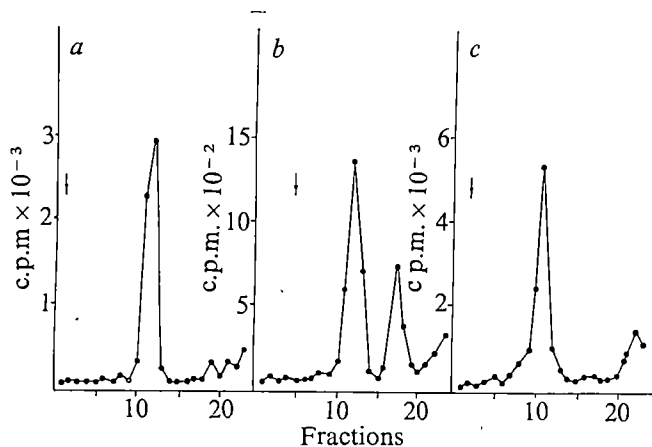
Fig. 1 Sedimentograms of virus-specific structures produced by the cultures chronically infected with measles (a), Sindbis (b) and tick-borne encephalitis (c) viruses. The cultures were treated with actinomycin D (2 μg ml⁻¹) at zero time, then ³H-uridine (20 μCi ml⁻¹) was added 1–3 h after treatment with the antibiotic. Thereafter the cells were disrupted in TNE buffer (Tris HCl 0.01 M pH 7.4, NaCl 0 1 M, EDTA 10⁻²) by a Dounce homogeniser, the nuclei and mitochondria were removed by centrifugation (15,000g, 20 min), the cytoplasmic extracts were layered on sucrose density gradients 15–40% (w/v) prepared in TNE buffer, centrifuged in a SW 27.1 rotor of a Spinco L 3-50 centrifuge at 122,500 r.p m. for 1 h 45 min (a) of 2 h (b, c), and acid-insoluble radioactivity of the gradient fractions was determined in a Packard-Tricarb liquid scincillation counter. In parallel experiments VERO cells were infected with measles virus, chick embryo fibroblasts with Sindbis virus, and SPEV (swine) cells with tick-borne encephalitis virus. The cultures were grown without actinomycin D, labelled with ³H-uridine (10 μCi ml⁻¹) overnight; the viruses were collected from the culture medium; the cell debris removed (15,000g, 20 min); the viruses were pelleted (120,000g, 2 h) and purified by equilibrium centrifugation (25,000 r.p.m. 16 h) in sucrose density gradients 20–60% (w/v) prepared in TNE buffer. The gradient fractions that corresponded to the density of viruses studied (1.22–1.24 g ml⁻¹) were collected and subjected to velocity centrifugation as indicated above. The arrows show sedimentation peaks of virions in the gradients parallel to a, b, and c.
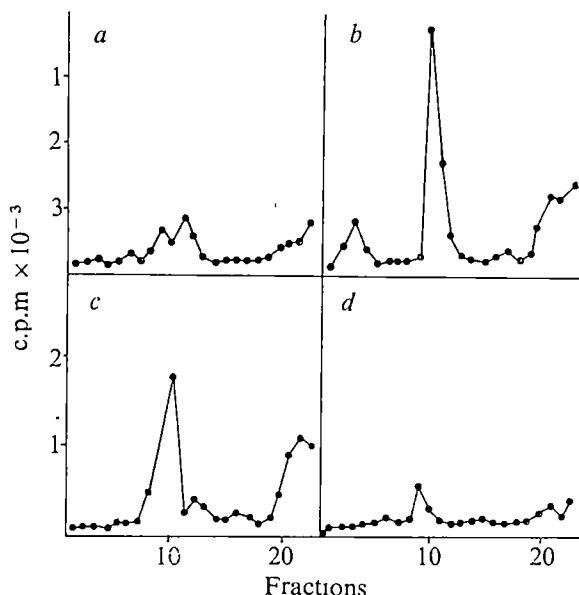
Fig. 2 Sedimentograms of virus-specific structures produced in SPEV cells acutely infected (a, b) and in Hep-2 cells chronically infected (c, d) with tick-borne encephalitis virus. SPEV cells were infected with the virus (10 PFU per cell), treated with actinomycin D (0.5 μg ml⁻¹) at zero time and incubated for 3 h (a) and 12 h. (b) ³H-uridine (20 μCi ml⁻¹) was added 1 to 3 h after infection (a) and 10 to 12 h (b) after infection. Hep-2-TBEV cells were treated with actinomycin D (0.5 μg ml⁻¹) at zero time and ³H-uridine was added 1 to 3 h (c) and 10 to 12 h (d) after treatment with the antibiotic. Cytoplasmic extracts were obtained and studied by velocity centrifugation in sucrose density gradients in the same way as described in the legend to Fig. 1.

cell line usually contain endogenous C-type oncornaviruses[13], Hep-2 cells have been reported to contain a new oncornavirus[14], and L cells are known to produce an endogenous C-type oncornavirus[15]. We carried out experiments to reveal latent oncornaviruses using a test that reveals reverse transcriptase activity associated with high molecular weight (60–70S) RNA[16] Such reverse transcriptase activity was shown in all three chronically-infected tissue cultures, and also in uninfected L and Hep-2 cells but not in uninfected primary chick embryo fibroblasts.

These results suggest that during the prolonged persistence of infectious viruses in cells containing latent oncornaviruses, the two types of viruses interact, resulting in the transcription of the infectious virus RNA genomes into double-stranded DNA which is then integrated into the genomes of the chronically-infected cell. The main molecular mechanism of virus persistence in these cultures is
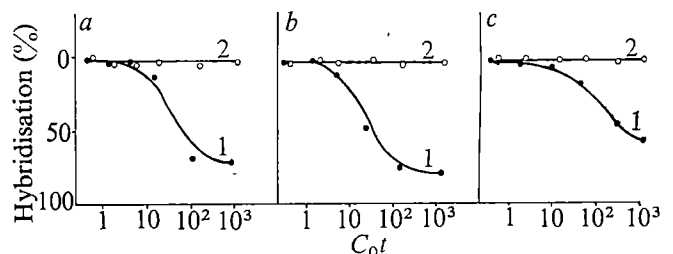
Fig. 3 Hybridisation of ³H-labelled RNAs of measles virus (a) with DNA from chronically infected (●) and uninfected (○) chick embryo fibroblasts; tick-borne encephalitis virus (b) with DNA from chronically infected (●) and uninfected (○) Hep-2 cells; Sindbis virus (c) with DNA from chronically infected (●) and uninfected (○) L cells The viruses were labelled with ³H-uridine, ³H–adenosine and ³H-guanosine (50 μCi ml⁻¹, 25 μCi ml⁻¹ and 25 μCi ml⁻¹, respectively) and purified as described in the legend to Fig 1 RNA was extracted with sodium dodecyl sulphate (0.5%) and phenol, precipitated with ethanol and stored at −20 °C Specific activity of the RNA preparations was 2 × 10⁵ to 6 × 10⁵ c p.m. μg⁻¹ and more than 80% of RNA sedimented in the region of 50S (measles) or 42–45S (Sindbis, tick-borne encephalitis). DNA from chronically-infected cells was extracted with phenol after treatment of the nuclear fraction of the cells with 0.2% SDS and Pronase (200 μg ml⁻¹) at 37 °C for 3 h. DNA was fragmented in an MSE ultrasonic disintegrator; RNA admixtures were hydrolysed by 0.5 M NaOH (37 °C, 16 h) with subsequent neutralisation; DNA was precipitated by ethanol and stored at −20 °C The hybridisation mixture contained in 0.1. ml: Tris HCl 0.02 M pH 7.4, NaCl 0.6 M, EDTA 10⁻³M, SDS 0.05%, RNA 2,000 c.p.m, DNA 1 5–2.5 mg ml⁻¹. The mixture (1 2 ml) was incubated at 68 °C; aliquots (0.1 ml) were taken at various intervals and stored at −20 °C to the end of the experiment. Then the samples were diluted with 3 ml of TN buffer (Tris HCl 0.01 M pH 7.4, NaCl 0 4 M), treated with pancreatic ribonuclease (50 μg ml⁻¹) at 37 °C for 1 h and acid-insoluble radioactivity was determined. RNA alone and RNA with calf thymus DNA incubated at the same temperature were taken as controls for self-annealing and nonspecific annealing. The corresponding radioactive counts (that were less than 100 c.p.m.) were subtracted and the % ribonuclease-resistant RNA–DNA hybrids were calculated and plotted against the corresponding C_0t values.

Table 1 Transfection of tick-borne encephalitis virus with DNA from Hep-2-TBEV cells

No. of experiments	Isolation of virus after treatment of SPEV cells with DNA from Hep-2-TBEV cells		
	Native DNA	DNA treated with RNase (300 μg ml⁻¹)	DNA treated with DNase (200 μg ml⁻¹)
1	10³ ⁵	10³ ¹	0
2	10³ ³	10³ ⁴	0
3	10³ ²	10³ ⁰	0

SPEV cells were treated with DEAE–Dextran (0 1%) for 10 min, then with DNA (200 μg) from Hep-2-TBEV cells, thereafter culture medium was added and the cultures were observed until cytopathic changes appeared. The figures indicate the titre of the virus (PFU ml⁻¹) in the culture medium. The specificity of the virus strains isolated was determined by plaque titration and neutralisation with immune serum and by intracerebral inoculation of mice.
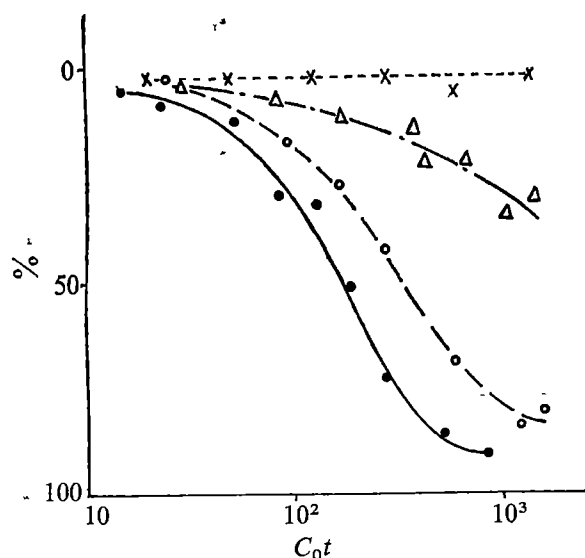
Fig. 4 Kinetics of hybridisation of ^{3}H–labelled measles virus RNA with DNA from leukocytes ($\triangle$), lymphatic node ($\bullet$), and kidney ($\bigcirc$) of a patient and of a fatal case of systemic lupus erythematosus and with DNA from normal embryonic human spleen ($\times$). The methods of preparation of ^{3}H-labelled measles virus RNA and of DNA from tissues as well as techniques of hybridisation were the same as described in the legend to Fig. 3. Hybridisation was carried out in the volume of 1.2 ml; aliquots (0.1 ml) were taken at various intervals and the percentage RNA hybridised was plotted against the corresponding $C_0 t$ values.

therefore the production of subviral structures, whose production is inhibited by the prolonged addition of actinomycin, by the cell genome, rather than the autonomous replication of virus particles, which are produced shortly after addition of the antibiotic.

As these models may be considered artificially induced, similar natural pathological processes were looked for. Systemic lupus erythematosus (SLE) provides a suitable study system. The disease is characterised by high titres of measles antibodies which correlate with the pathological course of the disease[17], by the presence of measles antigens in leukocytes detected by immunofluorescence[18], and by tubular structures in the cytoplasm of SLE-affected tissues resembling nucleocapsids of paramyxoviruses[19]. ^{3}H-labelled measles virus RNA was hybridised to DNA from tissues and leukocytes from SLE patients and fatal cases. Sequences homologous with measles virus were detected in the DNA from these tissues (Fig 4). No such sequences were detected in DNA from normal human tissues and in

DNA from leukocytes of measles patients taken at an early stage in the disease and in convalescence.

Tissues affected with lupus erythematosus and leukocytes from patients with the generalised form of the disease were assayed for reverse transcriptase activity associated with high molecular weight RNA. This test is positive with cytoplasmic extracts from lupus erythematosus tissues. In parallel studies we revealed tubular structures in lupus-affected tissues (electron microscopy) and measles virus antigens in leukocytes (immunofluorescence). These results suggest the following molecular mechanism for the initial events in the development of SLE.

In comparatively rare cases of infection with measles or measles-like virus, the virus interacts with a latent oncornavirus, resulting in transcription of the RNA of the infectious virus into double-stranded DNA and integration of the latter into the cell genome. The integrated viral genome may be expressed in the synthesis of virus-specific envelope proteins that, in the case of paramyxoviruses (including measles virus) are incorporated into the cell membrane and modify its antigenic properties[20]. Such cells become targets for attack by immunocompetent T cells and this triggers the development of the autoimmune disease.

Among human diseases similar molecular mechanisms may underlie syndromes closely related to lupus erythematosus (dermatomyositis, sclerodermy, periarteriitis nodosa) and also some chronic degenerative diseases of the central nervous system (subacute sclerosing panencephalitis, disseminated sclerosis).

1 Zhdanov, V. M., and Parfanovich, M. I , *Archs ges. Virusforsch.*, **45**, 225 (1974).
2 Zhdanov, V. M., *et al*, *Archs ges. Virusforsch*, **45**, 215 (1974).
3 Zhdanov, V. M , *et al.*, *Dokl. Akad. Nauk S.S.S.R.*, **218**, 616 (1974)
4 Parfanovich, M I., and Zhdanov, V. M., in *Molecular Biology of Viruses* (Moscow, 1973).
5 Azadova, N. B , Zhdanov, V. M., Kopelman, R H. and Gavrilov, V. I , *Vopr. Virusol.*, **17**, 520 (1972).
6 Zhdanov, V. M., Azadova, N. B., and Kopelman, R. N., *Vopr. Virusol.*, **18**, 524 (1973)
7 Zhdanov, V. M., Gavrilov, V. I., Klimenko, S. M , Bogomolova, N N., and Andzhaparidze, O G , *Experientia*, **29**, 235 (1973).
8 Zhdanov, V M , Gavrilov, V. I., Klimenko, S. M., Bogomolova, N. N., and Andzhaparidze, O. G., *Vopr Virusol.*, **18**, 17 (1973).
9 Zhdanov, V. M., Bogomolova, N N., Gavrilov, V. I., and Andzhaparidze, O. G., *Vopr Virusol.*, **18**, 23 (1973).
10 Zhdanov, V M , Gavrilov, V I , Bogomolova, N. N , Klimenko, S. M., and Andzhaparidze, O G. in *Molecular Biology of Viruses*, (Moscow, 1973).
11 Zhdanov, V. M , Gavrilov, V. I., Bogomolova, N. N , Klimenko, S. M., and Andzhaparidze, O. G., *Rev. roum Virol.*, **25**, 1 (1974).
12 Gavrilov, V. I. *et al*, *Archs ges. Virusforsch.*, **46**, 248–252 (1974)
13 Huebner, R J., and Todaro, G J , *Proc. natn. Acad. Sci. U.S A*, **64**, 1087 (1969).
14 Ilyin, K V , Bykovsky, A. F., and Zhdanov, V. M., *Cancer*, **32**, 89 (1973).
15 Dales, S., and Howatson, A F., *Cancer Res.*, **21**, 193 (1961).
16 Schlom, J., and Spiegelman, S , *Science*, **174**, 840 (1971).
17 Philips, P E , and Christian, C. J., *Arth and Rheum.*, **12**, 688, (1969).
18 Alekberova, Z S., thesis (Russian), Univ Moscow (1973)
19 Fresco, B., *Fedn Proc* , **27**, 246 (1968).
20 Rott, R., and Schafer, W., in *Cellular Biology of Myxovirus Infection* (Foundation symposium) (Boston, 1964).

letters to nature

Definition of 'charge on an atom' and nature of the inductive effect

A PRECISE measure of the charge on an atom in a molecule is provided by calculating the number of electrons within a sphere of covalent radius using molecular wavefunctions. The results for alkyl substitution suggest that the simple qualitative ideas of the inductive effect may be less simple than is commonly supposed and that methyl groups are not intrinsic electron donors but rather attracters of charge.

Although the only precise 'charge on an atom' is its nuclear charge, chemists frequently use the expression for what is

more correctly the charge 'close to' an atom. Quantum mechanical calculations provide values of such quantities by using the so-called Mulliken Population Analysis[1]. This has the virtue of being easily programmed for a computer, but has the defect of assigning all the charge in a molecule to particular nuclei. Nonetheless it is the origin of most of the quoted 'charges on atoms' which appear in the literature, and its well known defects such as equal division of charge between bonded centres and occasional negative populations are usually ignored.

In principle, from a molecular wavefunction, ψ, it is possible to calculate the electronic charge in any restricted region of
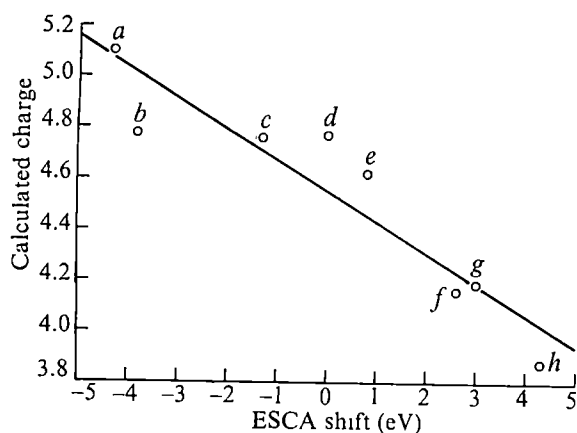
Fig. 1 Relationship between calculated charge within one covalent radius of nitrogen atoms and X-ray photoelectron shifts $(1s_N)$ for: a, NH_3; b, CH_3CN; c, $^*NNO$, d, N_2; e, NO; f, NN^*O, g, NO_2; h, NF_3.

space by integrating the value of $\psi\psi^*$ over the defined region. This precise definition of charge in a specified region has rarely been used[2,3] because the computation of the integrals over restricted regions of space is very difficult.

As part of our interest in the charge 'on' the nitrogen atom in the ammonium group of biologically active amines, we have overcome these integration problems by taking the interesting atom (nitrogen) as the centre of our polar coordinate system. The charge, q, within a sphere of radius r centred on the nitrogen atom is then given by:

$$q = \int_0^{2\pi} \int_0^{\pi} \int_0^{r} \psi\psi^* r^2 \sin\theta \, dr \, d\theta \, d\varphi$$

In these calculations, the approximation has been made that the charge around an atom arises only from the integral of the following terms in the square of the LCAO expression:

$$\sum_i \sum_j (c^2_i\chi^2_i + 2c_ic_j\chi_i\chi_j + c^2_j\chi^2_j)$$

where the is are atomic orbitals of the central atom, and the js are centred on atoms bonded to this central atom. This is an excellent approximation: terms from non-bonded atoms are very small.

Table 1 Calculated charges in substituted ammonium ions

Molecule	Charge on nitrogen atom
NH_4^+	5.054
$CH_3 \cdot NH_3^+$	4.774
$C_2H_5 \cdot NH_3^+$	4.786
$(CH_3)_3NH_4^+$	4.492
$CH_3 \cdot NH^+_2 \cdot C_2H_5$	4.501
$CH_3 \cdot NH_2 \cdot i\text{-}C_3H_7$	4.508
$CH_3 \cdot NH_2 \cdot n\text{-}C_3H_7$	4.503

For each molecule we have considered, good *ab initio* molecular wavefunctions have been calculated using the Gaussian '70 molecular orbital program[4] which takes account of all the electrons in the molecule, and overlap integrals have been calculated using the method of Barnett and Coulson[5]. The charge 'on' the nitrogen atom is defined as the electronic charge—the number of electrons—within one covalent radius (0.7 Å).

Charges were first calculated for small nitrogen-containing molecules for which X-ray photoelectron spectra (ESCA)

shifts are available[6]. The energy shifts for 1s-electron ionisation are thought[7] to depend in a reasonably direct way on the charge 'on' the atom, and thus provide a means of testing the physical significance of our calculated charges. Figure 1 shows that the correlation between charge and shift is good. The Mulliken charges, in spite of the simple basis on which they are computed, give a correlation with shift which is only slightly less good.

As model compounds of particular interest to us we considered substituted ammonium ions. The calculated charges are shown in Table 1. The fact that the charge on the nitrogen atom decreases with increasing substitution of hydrogen atoms by alkyl groups is particularly significant and is in direct opposition to the traditional view that alkyl groups have a $+I$ effect—that they donate electrons, relative to hydrogen.

Much of the evidence from which the $+I$ effect of alkyl groups is inferred pertains to reactions and equilibria in solution: the present calculations refer to molecules in the gas phase, and so comparisons with solution data must be made with caution. But some gas-phase data are available; the most directly relevant concern dipole moments.

Laurie *et al.*[8] have performed a series of microwave measurements of dipole moments. They have shown that the dipole moment of propane is $0.085D$ with the electron drift in the direction shown:.
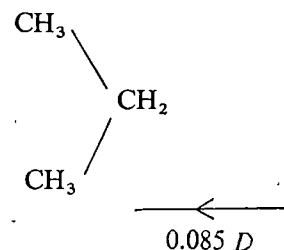
This very strongly suggests that methyl groups are in fact 'electron-withdrawing' relative to hydrogen atoms.

The dipole moments of a series of chloroalkanes are shown in Table 2. Assuming that the dipole acts in the direction $R \rightarrow Cl$, the electron-withdrawing power of the different alkyl groups, R, may be inferred to be:

$$CH_3 > C_2H_5 > n\text{-}C_3H_7 > i\text{-}C_3H_7$$
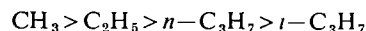
This is also the order inferred from the calculated charges in ammonium ions.

Spectroscopic measurements suggest that in certain circumstances alkyl groups may act as electron acceptors rather than as electron donors[9]. The principle electronic transition in phenol, anisole, aniline and dimethylaniline in the gas phase may be presented crudely as·
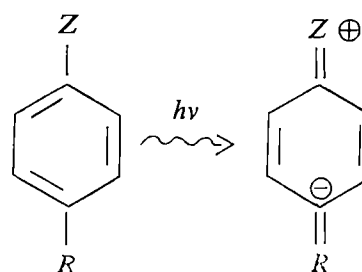
Solvent effects confirm that the electron migration in these transitions is indeed away from the functional group Z and towards the *para* substituent R. Now when R is an alkyl group, the energy of the transition is lower than when R is a hydrogen atom· thus the alkyl group seems to act as an electron acceptor.

Table 2 Dipole moments of chloroalkanes[10]

Molecule	Dipole moment (D)
$CH_3 \cdot Cl$	1.83
C_2H_5Cl	2.00
$i\text{-}C_3H_7Cl$	2.15
$n\text{-}C_3H_7Cl$	2.04

Ingold[10] and Braumann[11,12] have suggested a reason why alkyl groups should seem to donate electrons in many experimental situations. The gas-phase basicities of ammonium derivatives increase in the order:

$$NH_2 < CH_3 \cdot NH_2 < (CH_3)_2NH$$

This is often quoted as evidence for the $+I$ effect of methyl groups. But Braumann et al.[11,12] have measured the gas-phase acidities of the same compounds, and have found that these increase in the same order. The $+I$ effect cannot explain both these sets of observations. Alkyl groups can, however, be polarised much more than hydrogen atoms, they are thus more able to stabilise both anions and cations, and therefore enhance both acidity and basicity.

Work by Hudson et al.[13] has placed this polarisability effect on a somewhat more quantitative basis by use of a perturbation model of alkyl substitution. The influence of the alkyl group is considered to be split into two parts, one a repulsion which is greater in the neutral molecule than in the ion, and one an attraction which acts only in the ion. This treatment predicts that alkyl groups in alcohols or amines may act as either apparent donors or acceptors, depending on the conditions.

Perhaps the area in which the idea of alkyl groups as electron donors has been most widely used is in the explanation of the activating, ortho–para directing effect of one or more alkyl groups introduced into benzene. Preliminary calculations suggest that, although the p-toluene tetrahedral intermediate involved in electrophilic substitution is indeed lower in energy than the meta-substituted intermediate, the charge distribution is unlike that predicted by simple resonance bonding theory. But the situation is far from simple and it is possible that solvation effects are very important.

Susan M. Dean
W. G. Richards

Physical Chemistry Laboratory,
University of Oxford,
South Parks Road, Oxford, UK

1 Mulliken, R S , *J. chem Phys* , 23, 1833 (1955)
2 Pollak, M , and Rein, R.,'*J. chem Phys* , 47, 2054 (1967)
3 Politzer, P , and Harris, R. R , *J. Am. chem Soc* , 92, 6451 (1970).
4 Hehre, W J , Stewart, R. F., and Pople, J. A , *J chem Phys* , 51, 2657 (1969)
5 Barnett, M. P , and Coulson, C A., *Phil. Trans R. Soc* , A243, 221 (1950)
6 Finn, P , Pearson, R K., Hollander, J M , and Jolly, W L , *Inorg. Chem* , 10, 378 (1971).
7 Siegbahn, K , *et al.*, *ESCA applied to free molecules* (North-Holland, Amsterdam, 1969)
8 Laurie, V W , and Muenter, J. S , *J. Am. chem. Soc* , 58, 2883 (1966), *J. chem Phys* , 45, 825 (1966).
9 Schubert, W M , Murphy, R B , and Robins, J., *Tetrahedron*, 17, 199 (1962)
10 Ingold, C K , *Structure and mechanism in organic chemistry*, 2nd ed (Bell, 1969).
11 Braumann, J I , Riviros, J. M , and Blair, L K , *J Am chem Soc* , 93, 3914 (1971)
12 Braumann, J I , and Blair, L K , *J Am chem Soc.*, 91, 2126 (1969)
13 Hudson, R F., Eisenstein, O , and Ank, N T , *Tetrahedron*, 31, 751 (1975)

A low velocity zone underlying a fast-spreading rise crest

We present the results of an unreversed seismic refraction profile on the East Pacific Rise near the Siqueiros Fracture Zone recorded using a digital ocean bottom seismograph (OBS)[1]. An analysis of P wave arrival times and amplitudes indicates a velocity gradient in the top 2 km, with the velocity reaching 6.7 km s^{-1}. This is underlain by a low velocity channel some 1.4 km thick in which the velocity decreases to around 4.8 km s^{-1}. Below this low velocity region there is a velocity gradient from 6.2 to 6.8 km s^{-1} and mantle velocities of 7.7 km s^{-1} are reached at a depth of 6 km below the sea bed.

Many surveys have been made of the structure in the neighbourhood of slow spreading ridges, particularly the Mid-Atlantic Ridge, but very little work has been done on fast spreading ridges. Two surveys[2,3] on the East Pacific Rise near the area of this work show mantle velocities below 8 km s^{-1}.

During the course of a gravity and seismic refraction survey in the neighbourhood of the Siqueiros Fracture Zone (Fig. 1)
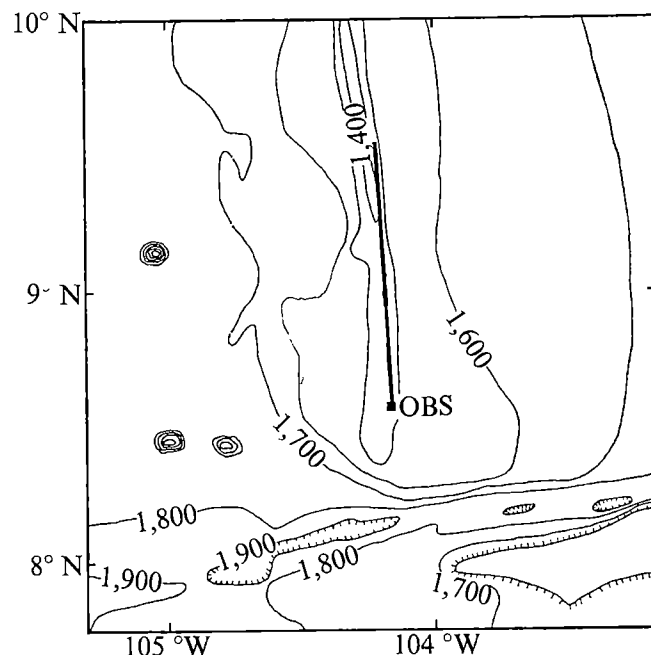
Fig. 1 Position of OBS refraction profile along the crest of the East Pacific Rise Contours are in uncorrected fathoms.

made with the Hawaii Institute of Geophysics, a refraction profile was carried out along the crest of the East Pacific Rise, all shots being recorded on a single OBS. The local spreading half rate is high, about 60 mm yr^{-1}, and the portion of the rise crest studied consists of a horsted block about 10 km across.

A record section of this ridge crest profile is shown in Fig. 2. A reduction velocity of 8 km s^{-1} has been applied to the section and, to facilitate amplitude studies, the traces have been normalised to a shot size of 109 kg (ref. 4). Since all shots were recorded with the same instrument, there is no problem with variability in instrumental response. The records have been passed through an aliasing filter in the OBS before digitising at 128 samples s^{-1} and subsequently bandpassed with a zero phase 1–9 Hz filter. Timing corrections have been made for the depth of the shot and bottom topography near the shot points.

The travel times (T) for both first arrivals and prominent second arrivals were measured for each distance (X). The graphical construction technique of Bessonova et al.[5] was then used to determine the delay time τ ($= T - pX$) for the values of the ray parameter p ($= dT/dX$) present in the records. A smoothed spline was also fitted to the (T,X) data and estimates of the slope p made from this, τ values were then found by direct construction. The values of $\tau(p)$ obtained from the two methods were in good agreement. The ray parameter, or inverse phase velocity, p corresponds to the local slope of a travel time curve and the delay time $\tau(p)$ to the time intercept at zero distance made by a tangent to the travel time curve with this slope.

The advantages of working with the $\tau(p)$ relation are that the delay time τ is a monotonically decreasing function of the ray parameter, and that for a model containing a low velocity zone

it exhibits a discontinuity for p equal to the reciprocal of the velocity at the lid of the zone. In the present case the τ/p curve we have determined has two distinct branches separated by a jump of 0.4 s between the values for phase velocities of 6.70 km s^{-1} and 7.00 km s^{-1}, which suggests the probable existence of a low velocity zone with a lid velocity of 6.7 km s^{-1}. Analysis of sonobuoy records at short range taken on the same profile give independent evidence for a velocity of 6.7 km s^{-1}.

From our delay time results we estimated upper and lower bounds on $\tau(p)$ and then inverted these to give extremal velocity profiles using the method of Bessonova et al.[5]. With the adoption of an assumed lower bound on the velocity in any low velocity zone, we are able to prescribe upper and lower bounds on the depth at which a velocity can occur, outside of a low velocity zone. Travel times for models which satisfied these bounds were tested against the travel time observations, and successful models were used in the amplitude calculations. Synthetic seismogram sections were calculated for these velocity profiles using a modification of the reflectivity algorithm[6,7] which enables the attenuation structure to be included. Although a large number of models could be found to fit the travel times, the relative amplitude of arrivals along the records proved an effective discriminant.
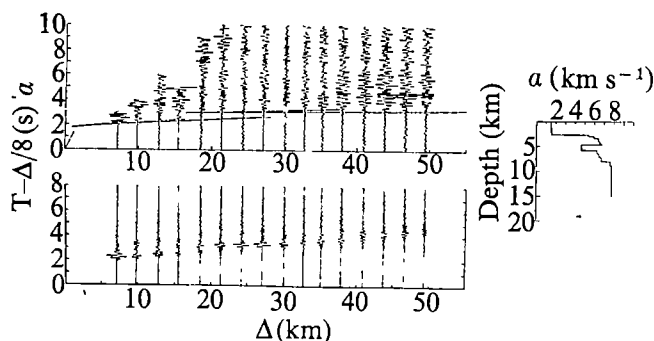
Fig. 2 Seismic profile and interpretation. Top figure shows the data with travel time curve corresponding to model at right. Bottom figure shows synthetics corresponding to model.

The model presented in Fig. 2, together with the corresponding synthetic seismogram section, was the best fit to both the travel time and amplitude criteria. Several attempts to model the observed records without a low velocity zone proved singularly unsuccessful with regard to the amplitude criteria. Models without low velocity zones also yielded crustal thicknesses significantly greater than any measured in ocean basins. We have assumed that all attenuation arises in shear[8] and adopted a shear wave Q of 175 in each of the layers, except in the low velocity channel where it was dropped to 100, since this improved the fit to observations. Unfortunately, we have no independent evidence to determine attenuation values. It should be noted that the amplitude of the first arrival between 30 and 50 km decreases too slowly for the transition to a 7.7 km s^{-1} layer to be a discontinuity, but the behaviour can be modelled quite well with a gradient. Even our best model does not fit the amplitude data exactly. Factors contributing to this difficulty in fitting the data include the inexactness of the normalisation and the likelihood of lateral variations along the profile.

The low velocity, lower Q zone beneath the ridge crest is hypothesised to be a zone of partial melt or a magma chamber as predicted by Cann[9,10]. This low velocity material is underlain by high velocity crust, and there is evidence of a low velocity 7.7 km s^{-1} mantle. Our observation of a mantle arrival requires any intrusion zone to be narrower than about half a wave length (0.7 km) wide at the mantle interface. This low velocity mantle is in agreement with most previous work at spreading centres[11-13].

For profiles conducted on the flanks of the rise crest during the same survey, mantle velocities in excess of 8 km s^{-1} were only detected when the crustal age exceeded 5 Myr.

The work was supported in part by the NSF; B.K thanks the Lindemann Trust for a fellowship.

JOHN ORCUTT
BRIAN KENNETT
LEROY DORMAN
WILLIAM PROTHERO

Scripps Institution of Oceanography,
University of California, San Diego,
La Jolla, California 92037

1 Prothero, W. A., *Bull. Seism. Soc. Am.*, 64, 1251–1262 (1974).
2 Shor, G. G , Menard, H. W., and Raitt, R. W., *The Sea*, 4, Part 2 (edit. by Maxwell), 3–27 (1970).
3 Snydsman, W , McClain, J., and Lewis, B., *EOS*, 56, 1147 (1974).
4 O'Brien, P. N. S., *Seismic Refraction Prospecting* (edit. by Musgrave), 152–170 (1970).
5 Bessonova, E. N., Fishman, V. M , Ryaboyi, V. Z., and Sitnikova, G. A., *Geophys J. R astr. Soc.*, 36, 377–398 (1974)
6 Fuchs, K., and Muller, G , *Geophys J. R astr. Soc*, 23, 417–433 (1971).
7 Kennett, B. L. N., *EOS*, 55, 1146 (1974).
8 Anderson, D L , and Archambeau, C. B , *J. geophys. Res.*, 69, 2071–2084 (1964).
9 Cann, J. R , *Nature*, 226, 928–930 (1970).
10 Cann, J. R., *Geophys. J. R. astr. Soc.*, 39, 169–187 (1974).
11 Le Pichon, X., Houtz, R. E., Drake, C. L., and Nafe, J. E., *J. geophys Res*, 70, 319–339 (1965).
12 Talwani, M., Windisch, C. D., and Langseth, M. G., *J. geophys. Res.*, 76, 473–517 (1971).
13 Keen, C., and Tramontini, C., *Geophys. J. R astr. Soc*, 20, 473–491 (1970)

Mercury contamination in a 54-m core from lake Huleh

THERE has been some discussion[1] concerning the suggestion of Aston et al.[2] that recent technological growth has resulted in a progressive increase in mercury in the sediments of Lake Windermere, UK. Here I report some data from another lake to substantiate their suggestion.

A 54-m core[3] was taken in the deepest portion of the now drained Lake Huleh located in the upper part of the Jordan Valley. The sediments are indicative of a swamp which underwent small periodic changes in level and was converted to a lake, presumably by the damming of the Jordan River by the late Pleistocene Yarda Basalt some 30,000 yr b.p. Before the great rise in water level reflected in the upper lacustrine series, there are two small lacustrine episodes when the water must have been transitorily too deep to deposit peat. Figure 1 shows the gross stratigraphy of the core.

The concentration of Hg in the Huleh sediments is highest in the surface mud of the modern lake (Fig. 1 and Table 1), whereas that in the second and first lacustrine periods is lower. Forty duplicate samples of each level ($n = 746$) were analysed for Hg by optical emission spectroscopy[4,5]. These results were confirmed by another laboratory using flameless atomic absorption spectroscopy. Mercury concentrations in the Huleh mud ranged from 2.36 to 0.11 p.p.m. with a mean of 0.36. The percentage relative s.d. for high values was 0.62 and that for low ones was 3.1 (ref. 4).

In the first 137 cm of the core, the Hg distribution follows that of Fe (ref. 6) (Fig. 1 and Table 2). The concentration of Hg begins to decline about 122 cm. From 142 cm to the beginning of the peat (4,572 cm) the distribution of Hg follows that of organic matter, here measured as percentage loss of weight on ignition[3]. There is a significant relationship between Hg and organic matter in the peat zones but the Hg concentration is more variable in this type of material. Although plant fragments prevalent in the more recent sediments may contribute to the Hg content[7], it would be presumed that this would also be the case in the peat zones, yet the latter contain sections where there is less Hg than in the standard lake mud.
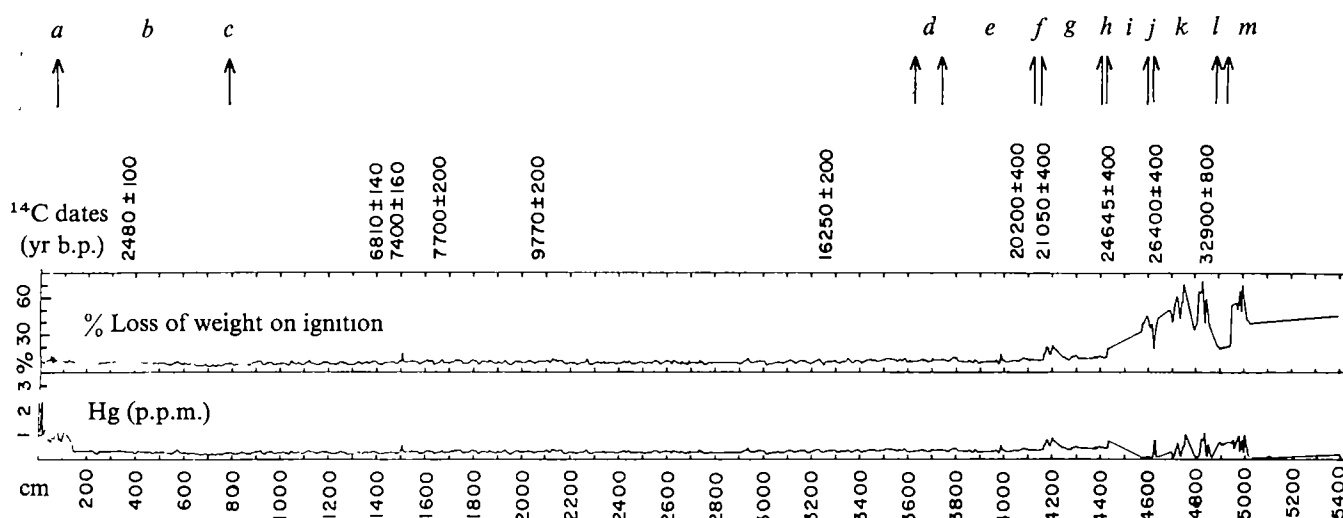
Fig. 1 Distribution of Hg and percentage loss of weight on ignition[3] in a 54-m core taken from Lake Huleh. Radiocarbon dates (organic C)[3] and the stratigraphy[3] of the core are included. *a*, Shallow water sediments with shell and some plant remains; *b*, grey stiff lake sediment with occasional shell; *c*, grey stiff lake sediment with shell largely absent; *d*, grey stiff lake sediment with decomposed clam shells; *e*, darker grey and more organic-looking material; *f*, beginning of main lacustrine episode, material less organic; *g*, peat with shell layers; *h*, second lacustrine period, dark grey sediment; *i*, peat; *j*, less peaty; *k*, peat with shell layers; *l*, first lacustrine period, very wet grey mud; *m*, peat with shell layers.

This would suggest that the plant fragments do not contribute significantly to the Hg content of the sediments.

It would seem on the basis of the linear correlation coefficients that Hg is initially associated with Fe and detrital clay particles. This has been noted in Lago Maggiore, Italy[8]. In Lake Huleh, for the whole core[9], quartz is associated significantly with halloysite ($r = 0.922$) and with generalised clay peak at 4.48 Å ($r = 0.884$) so that a correction for the diluting effect of quartz was not made[10]. The relationship between the mineralogical components of the core and Hg is significant only in the upper portion of the mud (Table 2).

Table 1 also shows the mean concentration of a few other elements that may reflect the effect of technological development. Both Ag and Cd are, in the absolute sense, more concentrated in the upper, more recently deposited mud than in the grey lake sediment beneath, although the mean rise in Ag is hardly significant. With Pb, however, the concentration at the surface is low because there is little automobile traffic in the vicinity of Lake Huleh. Presumably the oscillations in Pb reflect the natural erosion processes in the basin.

It has been suggested by some[1] and rejected by others[2,10] that upward postdepositional migration of Hg by organisms, or through interstitial waters by ionic or molecular diffusion, or compaction of the sediment, could produce a rise in Hg concentration in the upper portions of the more recently deposited sediment. As the surface muds of the earlier lacustrine periods do not show the increase in concentration so noticeable in the recent modern surface, such upward mobility does not seem to have been significant in the Huleh core.

On the basis of radiocarbon dates[3], the rise in Hg in the Lake Huleh sediments begins about 1200 AD. The first recorded mention of Hg was by Aristotle in the fourth century BC when it was used in religious ceremonies[11]. Until the sixteenth century[12], consumption was small and chiefly for medicinal and cosmetic purposes. Before 1850 (ref. 12), two great mines dominated European production. The Almaden mine in Spain began production in 400 BC and the Idria mine in Yugoslavia was first used about 1470. Besides such activity, heavy industry, the burning of fossil fuels and sewage disposal have all contributed to the global Hg distribution which eventually finds

Table 1 Mean concentration (p.p.m.) of some elements in various portions of the Lake Huleh core

Depth (cm)	Hg	Cd	Pb	Ag	Number of samples	Stratigraphy
0–77	1 07	1.66	94 9	0.069	15	Modern Lake mud surface
82–792	0.36	0.58	97 2	0.055	91	Grey Lake mud
4,245–4,425	0.48	0.78	113.5	0.054	25	Second lacustrine period
4,890–4,941	0.65	2.49	111.5	0 11	11	First lacustrine period

Table 2 Pertinent linear correlation coefficients between Hg and other substances[7,9] in the Lake Huleh core

Depth (cm)	Montmoril-lonite	Halloysite	4.48 Å peak	Quartz	Calcite	Fe	% Loss of weight on ignition
0–77	0.640*	0.918	0.922	0.910	−0.852	—	—
0–137	—	—	—	—	—	0.939	−0 221
137–792							0.987
Peat						−0.129†	0.956
0–5,400							0.194
Without peat							0.562
Without peat and 0–137 cm							0.982

*Not significant, †, 2% level.
Unless otherwise noted all are significant beyond the 0 1% level.

its way to the atmosphere. Such information and the data obtained from the analyses of the Lake Huleh mud certainly suggest that the increase in Hg concentration in recently deposited lake muds reflects man's global activities.

I thank the National Science Foundation for support and Dr F. Breck of the Jarrell–Ash Company, Waltham, Massachusetts for Hg analyses.

U. M. Cowgill

Department of Biology,
University of Pittsburgh,
Pittsburgh, Pennsylvania 15260

1 Sikes, C. S., and Drain, M. P., *Nature*, **244**, 529 (1973).
2 Aston, S. R., Bruty, D , Chester, R., and Padgham, R. C., *Nature*, **241**, 450–451 (1973).
3 Cowgill, U. M., *Archs Hydrobiol.*, **66**, 249–272 (1969).
4 Cowgill, U. M., *Devl appl. Spectrosc.*, **10**, 331–352 (1972).
5 Cowgill, U. M., *Appl. Spectrosc.*, **28**, 455–457 (1974).
6 Hutchinson, G. E., and Cowgill, U. M., *Archs Hydrobiol.*, **72**, 145–185 (1973).
7 Cowgill, U. M., *Archs Hydrobiol. suppl.*, **45**, 1–119 (1974).
8 Damiani, V., and Thomas, R. L., *Nature*, **251**, 696–697 (1974).
9 Cowgill, U. M., *Archs Hydrobiol.*, **71**, 421–474 (1973)
10 Thomas, R. L., *Can. J. Earth Sci.*, **9**, 636–651 (1972); **10**, 194–202 (1973).
11 Agricula, G., *De Re Metallica* (1556), (trans. by Hoover, H. C., and Hoover, L. H.), 432 (Dover, New York, 1950).
12 Bureau of Mines, *Mineral Facts and Problems*, 639 (Bureau of Mines, 1970).

A resonant point absorber of ocean-wave power

Various large scale systems have been proposed for absorbing and utilising the energy carried by ocean waves[1-4]. In principle, such a system consists of a damped oscillator which interacts with the wave. The net result is that energy is transferred from the wave to a load. Here we call a system a 'point absorber' when its horizontal extent is much smaller than one wavelength, and a 'linear absorber' when the system is made as a straight construction, at least a few wavelengths long[1].

A progressive wave on deep water transports an energy of

$$K = (\rho g^2/2\omega)\ \langle \eta^2 \rangle$$

per unit time and per unit length of the frontage of the wave. Here, ρ is the density of water, g is the acceleration of gravity, η is the surface elevation and ω is the angular frequency of the wave. $\langle \ \rangle$ Represent average over a time which is typically 20 min.

For the region west of the Hebrides Salter[1] reports that K has an annual average of 77 kW m^{-1}. Although the power incident on the coast may be somewhat smaller, ocean waves represent a very interesting energy resource for the coastal countries if the energy can be converted at a reasonable cost.

We report here on a proposed point absorber which is optimised for efficient energy conversion[3]. The resonant frequency of such an absorber is at all times tuned to the characteristic frequency of the wave. This may be realised by incorporating into the oscillating system a flywheel with adjustable moment of inertia. The importance of this tuning facility is that at resonance, the movement of the oscillator is in phase with the dynamic pressure of the incoming wave, resulting in a substantial transfer of energy from the wave to the oscillator. At resonance the motion of the oscillator is magnified with respect to the motion of the incoming wave. So the oscillator is able to produce the oscillating fluid displacement which is required for an effective interaction between the wave and the point absorber. Thereby, in a very efficient way, a secondary, ring-shaped, outgoing wave is generated, which interferes with the incoming wave in such a way that the resulting transmitted wave carries with it less energy than the incoming wave does.

Another condition which must be satisfied to obtain optimum performance concerns the energy delivery from the resonant oscillator. If the waves are sinusoidal, the condition for optimum

power output is that equal amounts of energy are delivered to the load and to the generated outgoing waves. This means that the mechanical load resistance should at all times be adjusted equal to the radiation resistance. But for wind-generated waves, which are not purely sinusoidal, the load resistance should be somewhat larger than the radiation resistance[3] to obtain maximum power output.

Resonant point absorbers having horizontal oscillating movement can be constructed, as can absorbers with vertical movement. As an example, consider the cylindrical, vertically oscillating tank of diameter $2a$ shown in Fig. 1. When the
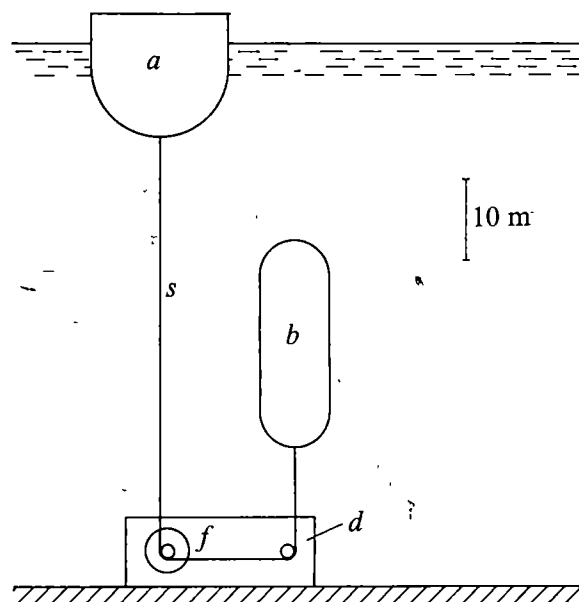
Fig. 1 An example of a single unit of a point absorber. The tank a is kept in a semi-submerged equilibrium position by means of the wire s which is stretched by the buoyancy of the auxilliary tank b. The wire s also drives a flywheel f so that it rotates in one direction when the tank a moves downwards and in the opposite direction when the tank moves upwards. The resonance frequency of the point absorber is tuned to the characteristic frequency of the wave by adjusting the inertia moment of the flywheel f which is placed in a housing d on the bottom of the sea.

resonant frequency is tuned to the frequency of the wave and the damping is optimised, then the maximum available useful power in small amplitude sinusoidal waves is[3]

$$P_{opt} = [(\rho g \pi a^2)^2/4R_r]\ \langle \eta^2 \rangle$$

Here R_r is the radiation resistance, and $\langle \eta^2 \rangle = \eta_0^2/2$ where η_0 is the amplitude of the wave.

We define an absorption length d_a by

$$P_{opt} = Kd_a$$

Here d_a expresses the width of a wave front across which passes an average amount of power equal to that converted by the point absorber. For the shallow tank in Fig. 1 we find that in sinusoidal waves of period of the order of 10 s the absorption length exceeds 50 m even though the diameter of the tank is only 16 m. But because of the power limit of the mechanical absorber and the corresponding electric generator, and because of the fact that wind-generated waves are not purely sinusoidal, the effective absorption length is smaller. With a reasonable power limit and a wave power spectrum with relative half-value width of 0.2, we obtain[3] an effective absorption length of

approximately 20 m as an annual average for waves typical of the North Atlantic.

The functioning of a point absorber is analogous to the operation of the antenna of a radio receiver. Such an antenna absorbs much more power from the wave, to which frequency it is tuned, than is incident on to its physical cross section. Correspondingly, the tuned oscillating tank has an absorption length larger than its diameter.

In contrast, a linear absorber has an absorption length which is, obviously, smaller than its physical extent. Reports on the linear absorber proposed by Salter indicate[5] an absorption length which exceeds 50% of the length of the construction.

Since the wave energy is available in a relatively narrow spectral frequency band, the resonant point absorber is a very effective energy converter. Its physical dimensions are not prohibitively large for mooring the device. The mooring provides a steady reference against which the oscillator can act with very good efficiency.

A practical construction of a resonant point absorber will probably be different from that shown in Fig. 1. It is possible to design a system where the flywheel and the other machinery are placed inside the heaving tank. Except for a mooring wire and anchor, no installation is then necessary on the bottom of the sea. The resonant point absorber is preferably placed in an exposed location just off the coast. This condition requires that the depth is, perhaps, 50 m at the installation site.

The absorbed energy may be converted to electrical energy by a generator connected to the flywheel, and which then acts as the adjustable load resistance. Since the construction is moored, the electrical energy produced could be brought ashore by cables.

As indicated previously it is necessary that any wave energy absorber generates secondary, outgoing waves. It should be noted that practically all the volume of the heaving tank is used to displace fluid and thus to generate outgoing waves. In contrast, other proposed wave absorbing systems have a considerable amount of dead volume which does not participate in generating secondary waves.

The technological problems, including mooring, force transmission, and energy conversion seem to be manageable. A technological and economic study is, however, necessary before designing a real resonant point absorber.

If the aim is to optimise the ratio between the absorbed, converted energy and the energy in the waves incident on a coastline, the linear absorber proposed by Salter[1] is probably a better solution than an array of point absorbers. But if the aim is to convert a given amount of energy as cheaply as possible, we believe that a line of resonant point absorbers may be superior to a linear absorber. The previously mentioned, movement magnification in the resonant oscillator is probably an additional advantage when the technological details of the power converting system are being considered.

A large scale power station must consist of many, say 100, resonant point absorbers. If they are placed on a line with a distance of 100 m between neighbouring units, they will cover a coast line of 10 km. The individual units (each of 16 m diameter) will then operate practically independently of each other and absorb at least 20% of the wave power which is incident on the 10 km coastline.

K. BUDAR
J. FALNES

Institutt for eksperimentalfysikk,
N-7034 Trondheim—NTH, Norway

1 Salter, S. H., *Nature* 249, 720-724 (1974).
2 Kayser, H , *IEEE Ocean*, 1, 240-243 (1974)
3 Budal, K , and Falnes, J , *Mar Sci Commun.*, 1 (in the press)
4 Woolley, M , and Platts, J , *New Scient* , 66, 241-243 (1975).
5 Swift-Hook, D. T , Count, B. M , Glendenning, I , and Salter, S , *Nature*, 254, 504-506 (1975)

Climatic reversal in northern North Atlantic

OVER the European arctic and subarctic seas the atmospheric circulation has recently tended towards northerly airflow, with an accompanying climatic deterioration. Since the 1950s direct northerly outbreaks have swept the Norwegian–Greenland Sea with increasing frequency, adding almost every year, particularly in the 1960s, to the severity of the climate in areas as far south as the British Isles. This change has been associated with the establishment over Greenland in the early 1950s of a persistent ridge of pressure anomaly and with its subsequent maintenance and intensification (on average) throughout the late 1950s and 1960s. Compared with the 'normal' climatic period of 1900–39 this cell represented an increase of over 3 mbar in the mean annual pressure at sea level in Greenland over the period 1956–65, but was very much more pronounced during the cold season (November–March). During the winter quarter (December–February), the increase in the mean pressure at sea level in Greenland amounted to over 7 mbar between 1900–39 and 1956–65 (Fig. 1a) and the pressure rise continued during the late 1960s. (Throughout, listed dates refer to the second year of the winter in question; thus, "winter 1956" refers to the period December 1955–February 1956.) A further increase of over 5 mbar occurred in Greenland between the winters of 1956–65 and 1966–70 (Fig. 1b). Coupled with a slight decrease of pressure over the eastern Norwegian and Barents Seas this change has resulted in ". . . a remarkable difference of pressure between Greenland and the eastern Norwegian Sea; this has increased since 1950 in every month of the year . . ."[1].

The resulting increase in the occurrence of northerlies over the Norwegian–Greenland Sea, and the physical repercussions, have been reviewed by Dickson and Lamb[2] and by Rodewald[3]. First, the great boosting of northerlies during the winter months resulted in a steep decline in the mean winter air temperature throughout this sector, especially in those areas lying to the north and west of the atmospheric and oceanic polar fronts. South from the high arctic, towards the British Isles, the winter cooling becomes rapidly less marked but there is evidence that since the early 1960s the resulting decline in sea temperature on the European shelf during the critical spring months (March–May) has been sufficient to affect (favourably) the survival of North Sea cod, which in those latitudes are approaching the equatorward limits of their range[4].

Apart from those effects, the strengthening of the northerly airstream has been responsible for a progressive southward extension of sea ice across the Greenland Sea, reaching a maximum extent in the spring of 1968. Icelandic observations[5] suggest that this change took place in two stages. First, as the northerlies increased in strength, the hydrographic character of the cold east Greenland and east Icelandic currents was altered so that they became cooler and less saline as the proportion of polar water increased. The east Icelandic current, which had been an ice-free arctic current during 1948–63, became a polar current in 1964–71, transporting and preserving drift ice.

Ancillary biological changes have been attributed to this climatic deterioration. They include an economically important alteration of the annual feeding migration of the Atlanto–Scandian herring stock to Iceland[2,6] and a delay in the spring production of phytoplankton in the southern Norwegian Sea (ref. 2, and G. A. Robinson, unpublished); a two-week reduction in the length of the growing season in England has also been reported during this period of cooling[7].

In the winter of 1970–71, the striking climatic trends evident over the Norwegian–Greenland Seas came to an abrupt halt. The high-pressure anomaly cell over Greenland, which had for so long dominated the patterns of climatic behaviour in
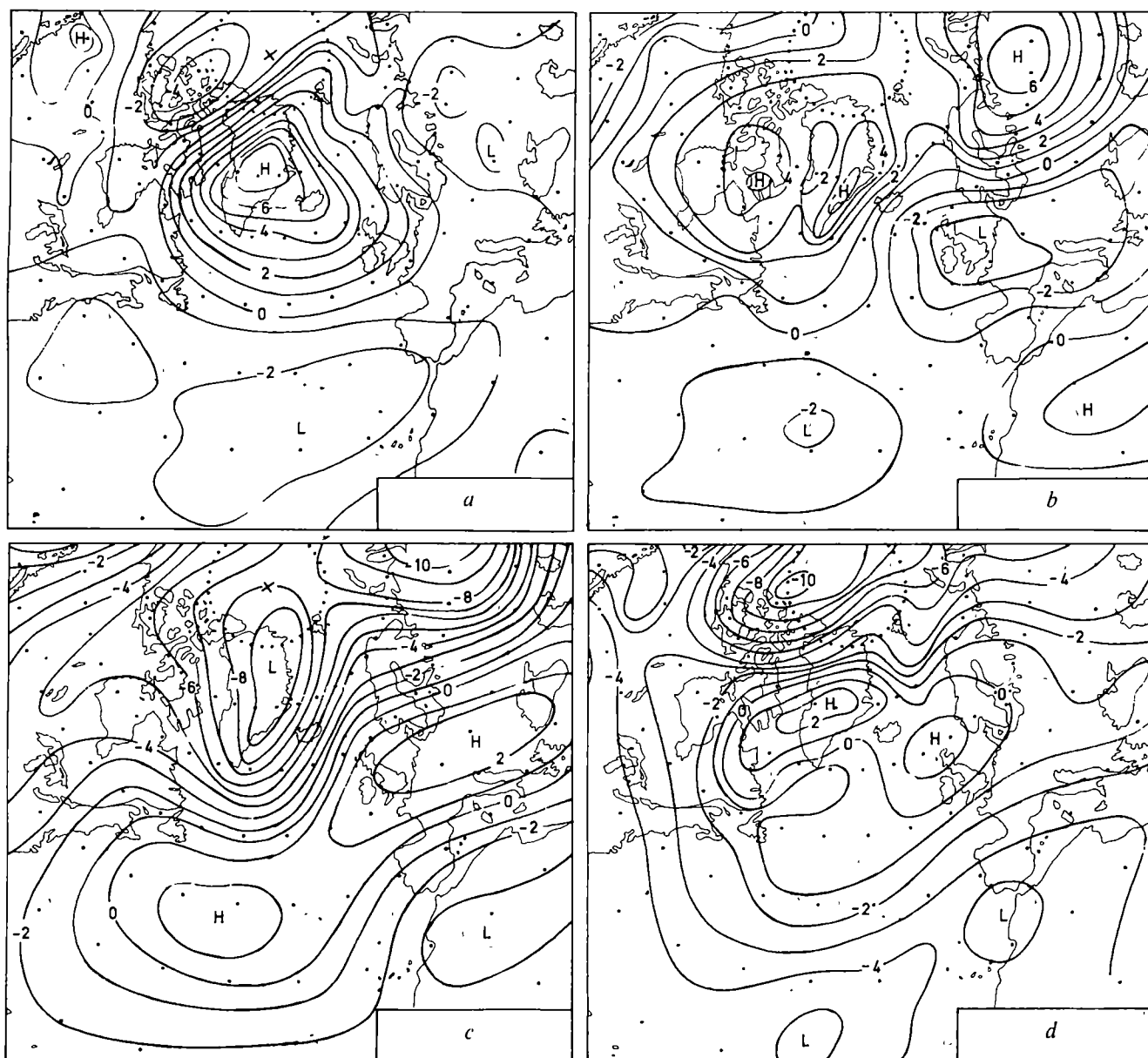
Fig. 1 Change of mean winter sealevel pressure (mbar) between the periods: *a*, 1900–39 and 1956–65; *b*, 1956–65 and 1966–70; *c*, 1966–70 and 1971–74; *d*, 1900–39 and 1971–74.

this sector, collapsed almost totally, and the mean pressure at sea level during winter fell by 9.6 mbar over Greenland between 1966–70 and 1971–74 (Fig. 1*c*). This ridge has not yet been totally eradicated, since in the earlier of the two periods the Greenland ridge had attained a peak intensity of over 12 mbar above the long term 'normal' (1900–39). Nevertheless, even though there is still a residual, high-pressure anomaly of some 2.8 mbar over Greenland (Fig. 1*d*) it is clear that the northerlies have been drastically weakened in the European arctic and subarctic since the winter of 1970.

The reason why such a persistent feature as the Greenland ridge should become weakened so drastically remains obscure, though the effect of the weakening is readily apparent in the succession of mild winters experienced over most of Europe since 1970. It is now also evident that this change has already brought about some amelioration in the marine climate off North Iceland. Fig. 2 illustrates the deviations of temperature and salinity from the 1950–58 mean, using observations made in June. Though conditions have not reverted to those of the mild 'arctic current' period (1948–63), both the temperature and salinity of the near-surface layer have risen since 1971 compared with the values obtaining during the 'polar current'
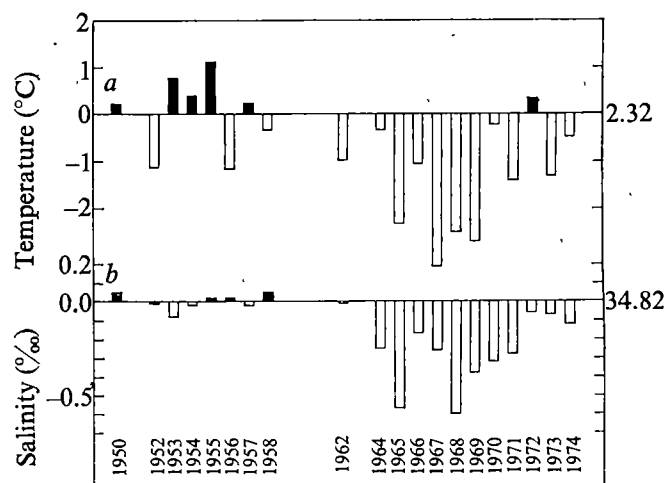
Fig. 2 Anomaly of temperature (*a*) and salinity (*b*) in June at a depth of 25 m in a study area between Iceland and Jan Mayen Island (67–69°N, 11–15°W; the averages for the normal period 1950–58 are shown).
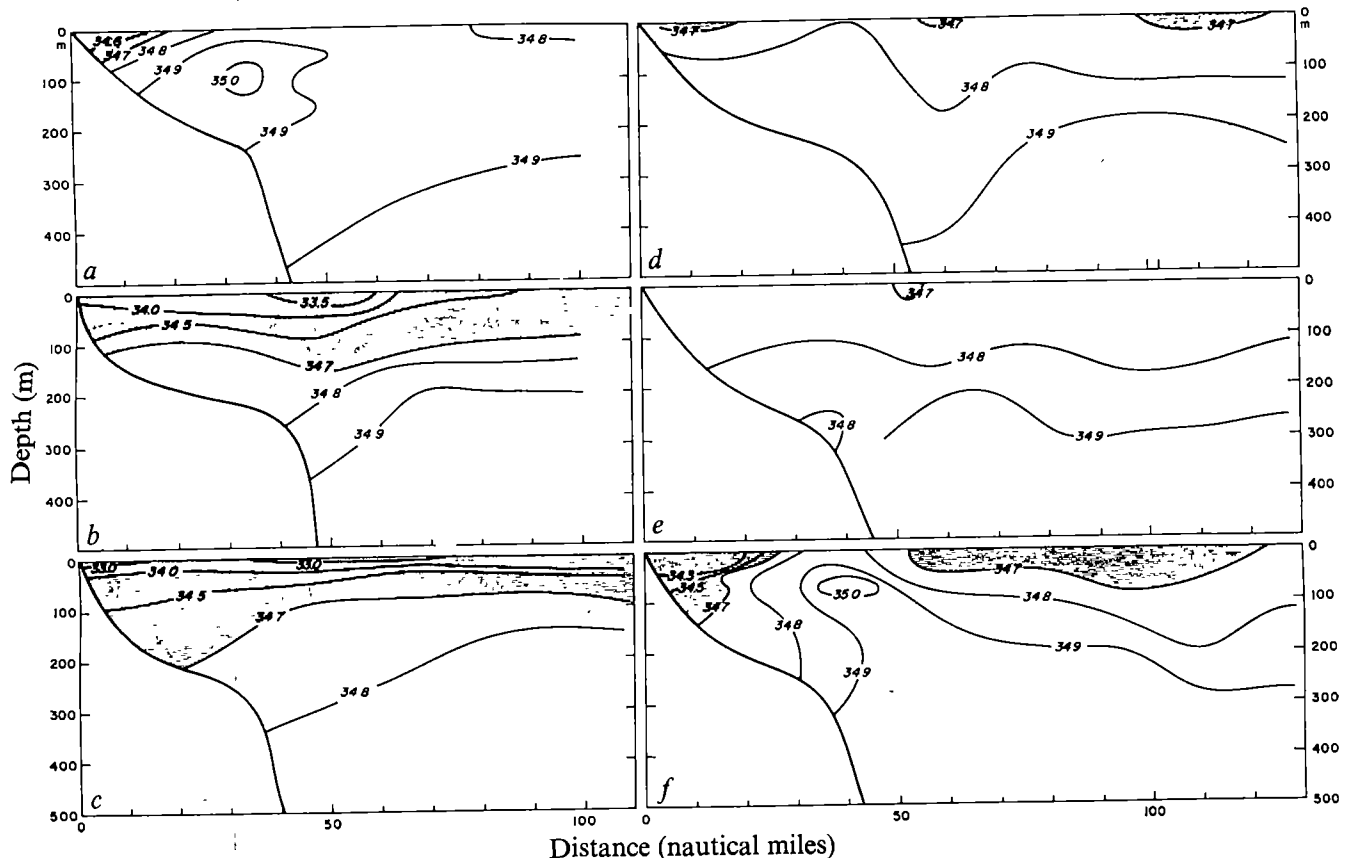
Fig. 3 Salinity distribution (°/oo) along a standard section worked north-eastwards from Langanes (north-eastern Iceland) in the month of June: 1957 (*a*), 1965 (*b*), 1968 (*c*), 1972 (*d*), 1973 (*e*) and 1974 (*f*).

period between 1964 and 1971. The increase in surface salinity is of especial importance in this area since, at salinities of more than 34.7‰, before the freezing point is reached, the surface layers will attain a sufficiently high density to over-turn and mix with the slightly warmer and more saline water at intermediate depths. At salinities of less than 34.7‰ the surface layers will not reach a sufficiently high density, even at the freezing point, to mix with these underlying layers and, instead, ice will tend to form.

Fig. 3 illustrates the recent weakening of polar influence off northern Iceland. The salinity section for June 1957 (Fig. 3*a*) is representative of a normal year during the relatively warm arctic period; 1965 (Fig. 3*b*) and 1968 (Fig. 3*c*) reflect conditions during the cold polar period (which reached its most extreme development in 1968) and the profiles for 1972–74 (Fig. 3*d–f*) represent conditions during the relatively mild period of the most recent years. As shown, polar water with a salinity of less than 34.7‰ was almost completely absent from the section in June 1972 and 1973, and though some renewed increase of polar water was observed in June 1974 its distribution was more restricted than in the 'polar current' period, 1964–71. Signifi-cantly, sea ice has posed no problem to navigation in northern Icelandic coastal waters since 1971 (H. Sigtryggson, personal communication).

In general, it may be concluded[8] that during the spring months of 1972–74 the Atlantic influx into northern Icelandic waters was stronger than in any year during the period 1965–71 (though remaining slightly weaker than the earlier 1950–60 normal[9]). The polar water component to the north and north-east of Iceland was weaker in the spring of 1972–74 than in any year since 1963 and only slightly stronger than in the warm period of earlier years. Thus the 'little ice age' observed in northern Icelandic waters in recent years seems to have ended with an amelioration of the marine climate during 1972–74. (It should be stressed, however, that this conclusion is diagnostic rather than prognostic.)

Although these physical and economic repercussions were most conspicuous close to the oceanic polar front at northern Iceland, similar trends of hydrographic change were also observed throughout the Norwegian–Greenland Sea during this period[10]. On 14 standard hydrographic sections worked in June across the entire ice-free area of the Norwegian–Greenland Sea from the latitude of Spitsbergen to that of south-western Norway (~60°–80°N), a general cooling has been observed between the mid 1950s and the late 1960s, with a rapid reversion to positive temperature anomalies occurring in the early 1970s. These broad scale changes in ocean temperature are in parallel with the strengthening and weakening tendencies of the northerlies in this sector, and presumably reflect changes both in the heat loss from the sea to the atmosphere, and changes in the poleward transport of warm water by the principal Atlantic current branches.

R. R. Dickson

Ministry of Agriculture, Fisheries and Food,
Fisheries Laboratory,
Lowestoft, Suffolk, UK

H. H. Lamb

Climatic Research Unit,
University of East Anglia,
Norwich, Norfolk, UK

S.-A. Malmberg

Marine Research Institute,
Reykjavik, Iceland

J. M. Colebrook

Institute for Marine Environmental Research,
Plymouth, Devon, UK

1 Lamb, H H , *Weather*, **18**, 10 (1965).
2 Dickson, R. R., and Lamb, H. H., *ICNAF, Spec. Publ. no.* 8, 35–62 (1972).

3 Rodewald, M., *Dt. hydrogr. Z.*, 25, 97–117 (1972).
4 Dickson, R. R., Pope, J. G., and Holden, M. J., in *The Early Life History of Fish*, 69–80 (Springer, Berlin, 1973).
5 Malmberg, S.-A., *Jokull*, 19, 30–43 (1969).
6 Jakobsson, J., *Jokull*, 19, 134–145 (1969)
7 Lamb, H. H., *Nature*, 223, 1209–1215 (1969).
8 Malmberg, S.-A., *Annls biol*, *Copenh*, 30, 17–21 (1975).
9 Stefansson, U., *Rit Fiskideild.*, 3, 269 (1962).
10 Alekseev, A. P., and Penin, V. V., *Okeanologiya*, 13, 563–568 (1973).

Americium 242m in nuclear test debris

DIAMOND et al.[1] discussed the production, in a thermonuclear explosion, of isobars of mass numbers 239 and greater by neutron reactions on uranium, followed by β decay; much the same arguments seem to apply to phenomena in plutonium devices. Both cases, viewed in this way, suggest that mass number 241 will be represented by both ^{241}Pu and ^{241}Am, mass number 242 only by ^{242}Pu, and mass number 243 only by ^{243}Am. Such considerations neglect, however, the fact that weapons-grade plutonium is always more or less contaminated by ^{241}Am, and that this nuclide has an appreciable cross section (100 barn for thermal neutrons and slightly larger for epithermal neutrons) for neutron capture leading to ^{242}Am; about 10% of such captures yield ^{242m}Am, the others going to the ground state. It seems, then, that one should expect to find in the debris from nuclear explosions not just two, but all three, of the long lived americium nuclides: ^{241}Am, ^{242m}Am and ^{243}Am.

extremely low levels, it should be practicable to measure ^{242m}Am in a variety of other samples, by searching for ^{242}Cm that is supported by a longer lived precursor. Actually we have remeasured one sample contaminated by fuel-reprocessing waste, and found that about 3% of the ^{242}Cm it contained was supported in this way.

The conclusion that ^{242m}Am has been produced, measurably, in nuclear tests, has some interesting consequences:

The ^{242}Cm daughter supported by decay of ^{242m}Am, in addition to being readily measurable in most areas of high fallout, should have, as a consequence of its generation by two rapidly successive radioactive decays (^{242m}Am decays, with a half life of 152 yr to ^{242g}Am, which decays with a half life of 16 h to ^{242}Cm), unusual environmental mobility, separating from other fallout transuranics much as ^{234}U has been found to separate from ^{238}U and ^{235}U.

Since ^{242}Cm decays to ^{238}Pu, its presence represents not merely an unevaluated additional source of this transuranic, but of a moiety of ^{238}Pu that would be expected to be more mobile than the fraction of that nuclide produced in the original event.

In plutonium the content of ^{241}Pu is greater in proportion to the fraction of Pu that has come as byproduct from electric power generation[4,5]; ^{241}Pu will, however, be a less important constituent of breeder reactor plutonium[6]. These considerations suggest that americium 242m will be a significant component of the debris from future nuclear tests, or peaceful

Table 1 Concentrations in disintegrations per minute per 100 kg

	^{239,240}Pu	^{241}Am	^{242}Cm	^{244}Cm	^{242}Cm/^{241}Am
Sample A	216±11	37.8±0.7	0.21 ±0.02	0.01±0.004	0.54%
Sample B	18± 3	2.67±0.06	0.013±0.004	ND	0.49%

ND, Not detectable.

We have been led to this consideration by our finding of a small but readily measurable amount of ^{242}Cm in samples of seawater that had been contaminated by close-in fallout from an isolated, low yield nuclear test in 1962. The 60-1 samples had been sealed in polyethylene containers from collection until being opened for radiochemical analysis in 1974. Americium 241 was separated from other nuclides, and prepared for α spectrometry, by radiochemical procedures[2,3]; in this scheme curium isotopes accompany americium almost quantitatively. The americium plates from the samples in question were counted in a low-level α spectrometer for 8,000 min, in a special effort to detect any ^{244}Cm remaining from that produced in the test. Because the two samples represented different water depths, and thus different dilutions of the test debris, the concentrations of transuranic nuclides were very different, but the ratios among them were similar (Table 1).

α-Emissions of energy corresponding to those of possible naturally occurring contaminants were absent, confirming the cleanness of the radiochemical separation.

The ^{242}Cm cannot, after more than 25 half lives, represent curium originally in the samples; we believe there is little likelihood it represents laboratory contamination. Great care was taken in the radiochemistry, and we have never before seen ^{242}Cm in americium plates, except in samples expected to have this nuclide from waste disposal; also the uniformity of ^{242}Cm/^{241}Am ratio in two samples of different activity level argues against accidental contamination.

It seems to us necessary to conclude that the ^{242}Cm observed is the daughter of ^{242m}Am produced in the original nuclear event, probably by neutron capture of ^{241}Am. Direct observation of ^{242m}Am, at the concentrations indicated, would be quite impossible: detector backgrounds are too high for measurement either of its own X-ray emission, or of the β particles from its daughter ^{242}Am. Because detector backgrounds can be maintained, for solid state α spectrometry, at

nuclear explosions, although diminishing in proportion to the increasing use of breeder reactor plutonium.

We urge our colleagues in environmental radiochemistry to make a careful search for ^{242m}Am, both to establish baseline data describing its present distribution in nature, and to set limits on the influence of this nuclide on the environmental behaviour of its descendants, ^{242}Cm and ^{238}Pu.

VAUGHAN T. BOWEN
HUGH D. LIVINGSTON

Woods Hole Oceanographic Institution,
Woods Hole, Massachusetts 02543

1 Diamond, H, et al, *Phys. Rev*, 119, 2000 (1960).
2 Bojanowski, R, Livingston, H. D., Schneider, D. L, and Mann, D. R., in *Reference Methods for Marine Radioactivity Studies II, Transuranic Elements, Ruthenium, Iodine and Silver* (IAEA, Vienna, in the press)
3 Livingston, H. D., Mann, D. R., and Bowen, V. T, in *Analytical Methods in Oceanography* (Am Chem Soc., Washington, DC, in the press).
4 Darral, K. G, Hammond, G C M, and Tyler, J F. C., *Analyst*, 98, 358 (1973).
5 Livingston, H D., Schneider, D. L, and Bowen, V T., *Earth planet. Sci Lett*, 25, 361 (1975).
6 Volchok, H L, et al., in *Assessing Potential Ocean Pollutants*, 27 (Natn Acad. Sci., Washington, DC, 1975)

Tree remains in southern Pennine peats

RECENT work in Ireland[1-3], Wales[4], and south-west England[5] has demonstrated the coincidence in time of the onset of blanket peat accumulation with increased levels of prehistoric human activity in the vicinity; since many upland blanket bog areas in the British Isles have remains of trees embedded within the basal peat layers, it has been suggested that these tree remains represent a former forest cover, the deliberate clearance of which led directly to blanket peat formation. That is to say, the tree remains are assumed to immediately predate the oldest blanket peat layers.
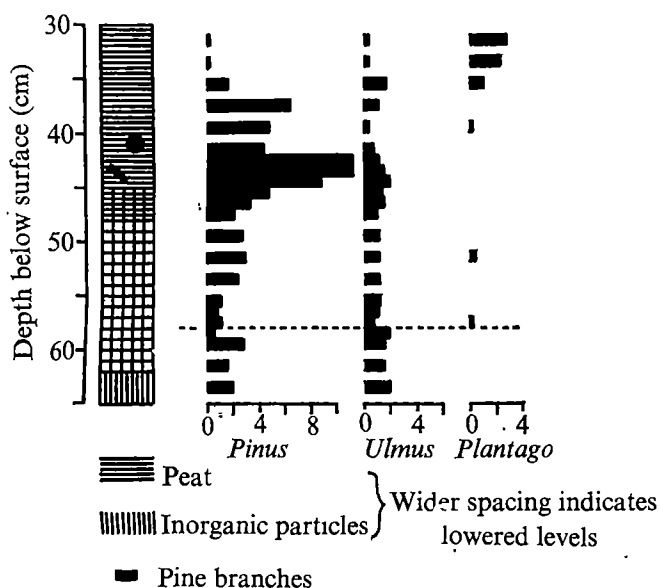
Fig. 1 Stratigraphy and selected pollen values for the basal peat layers at site 1, Lady Clough Moor, Yorkshire (at the blanket peat margin). The pollen values are expressed as percentage total land pollen. The broken line marks the position of the Elm Decline

Table 1 Records of tree remains in southern Pennine blanket peats

Species	No. of sites	Altitudinal range (m)
Birch (*Betula* spp.)	16	320–595
Pine (*Pinus sylvestris*)	15	350–500
Oak (*Quercus* spp.)	10	320–470
Alder (*Alnus glutinosa*)*	5	290–400
Willow (*Salix* spp.)†	4	400–490
Hawthorn (*Crataegus* spp.)	1	about 350
Hazel (*Corylus avellana*)	1	about 320

* Includes 4 records of 'alder/birch wood' by Hicks[11].
† Includes one tentative record by Conway[9].

In the southern Pennines tree remains (in the form of twigs, charcoal, larger branches, procumbent trunks up to 2 m in length, or trunk bases and roots) occur widely within or below the basal blanket peat layers at altitudes from 290–595 m. Table 1 summarises the data currently available for 36 known sites, in terms of tree species recorded and the numbers of sites and altitudinal ranges at which they occur. The data are derived partly from records in the literature[6-11], partly from personal records, and particularly from extensive field surveys carried out by D. W. Yalden. The sites vary considerably in extent, ranging from those comprising the isolated tree stump in a moorland area of several hectares to those with locally dense concentrations of stumps and wood fragments. The majority of the sites are close to the margins of the blanket peat, but this distribution may merely reflect the relative scarcity of situations elsewhere where deep peat faces are exposed down to the underlying bedrock (and thus where

basal tree remains can be observed). Tree remains are rarely recorded from altitudes above 500 m. Some sort of altitudinal zonation can be detected from the data in Table 1 for the four commonest species, with birch and pine remains predominant in the peat at the higher altitudes, and alder restricted to altitudes below 400 m. The widespread distribution of pine remains in the peat has not hitherto been recognised for the southern Pennines, and, indeed, pine remains have hitherto only been widely reported from Scottish blanket peats.

Several studies have shown that much of the blanket peat in the southern Pennines started to form in early Atlantic times, around 5000 BC; and accordingly it seems likely at first that these widespread pine remains represent relics of the pre-peat forests of Boreal times (as in Scotland[12]). But at the three sites so far investigated in detail, both pine and birch remains occur not at the soil–peat interface but actually within the basal peat layers. Although roots may extend downwards into the underlying mineral soil, the smaller twigs in particular are situated at distances of 5–20 cm upwards into the peat. Pollen diagrams from one of these sites (Lady Clough Moor, grid reference SK 105926, altitude 500 m) are shown in abbreviated form in Figs 1 and 2. At the peat margin on Lady Clough Moor, with peat depths of less than 1 m on slopes of up to 25°, pine remains are abundant in the peat, and coincide in position with a temporary peak of *Pinus* values in the pollen diagram (Fig. 1); farther back from the margin, on slopes of less than 10° and with deeper peat, birch remains are predominant, but the *Pinus* pollen peak is still present (Fig. 2). Clearly this *Pinus* peak can be referred to the Sub-Boreal period (pollen zone VIIb, about 3000–500 BC), since it occurs a short distance above the initial decline of *Ulmus* pollen values (the Elm Decline, shown
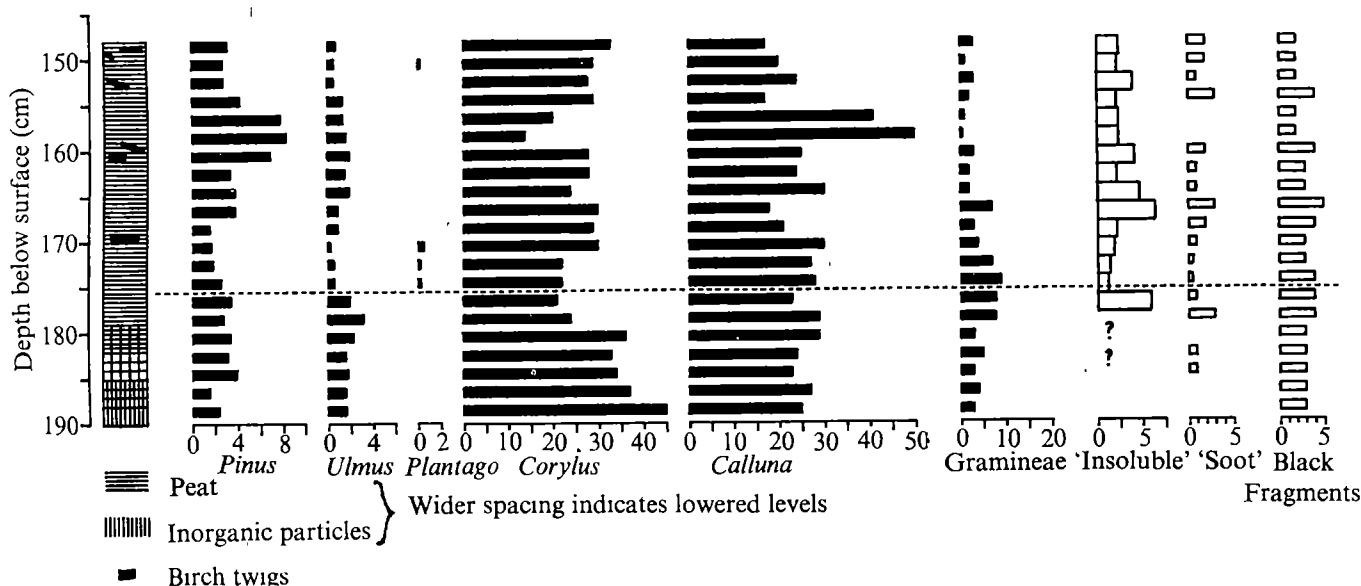
Fig. 2 Stratigraphy, selected pollen values, and estimates of carbon content for the basal peat layers at site 2, [Lady Clough Moor, Yorkshire (30 m in from the blanket peat margin). The pollen values are expressed as percentage total land pollen. The broken line marks the position of the Elm Decline

by a broken horizontal line in Figs 1 and 2) and the first appearance of occasional pollen grains of *Plantago*. Although more precise dating of the pine and birch remains is not yet available, it is possible that they could be as late as the uppermost alder/birch layers at Leash Fen[11], which have been radiocarbon dated at 1500–1790 BC. Without further extensive field surveys, pollen analyses, and radiocarbon datings it is clearly impossible to say whether this Lady Clough Moor site is typical, or whether some of the tree remains in the southern Pennines do indeed predate the inception of the peat blanket. There is no reason, however, to suppose that the site is not typical, and accordingly some mechanism has to be postulated whereby secondary colonisation of shallow blanket peat by trees became possible for a relatively short time during the Sub-Boreal period.

In the basal layers of all marginal peats so far investigated in the southern Pennines there is widespread evidence of burning —either in the form of microscopic carbon particles (similar to contemporary 'soot'), small charred plant fragments, or larger lumps of charcoal. Figure 2 shows some preliminary attempts to quantify this evidence of burning. The two right-hand columns show the abundance of soot-like particles and of charred plant fragments in the microscope slides prepared for pollen analysis, assessed subjectively on a five-point scale of frequency. The third column shows the weight of insoluble residue left after digestion of the peat with 20% potassium hydroxide and concentrated nitric acid (expressed as a percentage of the oven-dry weight of the peat). Under the microscope the majority of this insoluble residue seems to be carbonaceous. Carbonisation of the peat occurs in all samples examined, although there are clear differences in levels of carbon in different samples. Evidence of burning is least in the samples with high *Pinus* values, whereas high soot levels in the peat occur immediately above and below this *Pinus* pollen peak, and also immediately below the Elm Decline. Patterns of change in *Calluna*, *Corylus*, and Gramineae pollen values to some extent follow the evidence from burning patterns.

Probably the whole southern Pennine area is within the potential altitudinal range of tree growth, so that it is possible that before, and in the early stages of peat formation, colonisation by trees was prevented by recurrent fires. These recurrent fires could have been a natural phenomenon, but it is equally plausible that they were anthropogenic in origin. Numerous Mesolithic chipping sites, where flint or chert brought in from outside was worked into the characteristic artefacts, have been recorded from the southern Pennine uplands (see ref. 13 and references therein), and the area seems to have been a favoured seasonal hunting ground for Mesolithic populations for several millennia[13]. Most of these Mesolithic sites are clustered around the present-day blanket peat margins, at altitudes of 360–480 m. At two of these sites pollen analyses have shown considerable local disturbances in the vegetation at the level of Mesolithic occupation[13]. But the widespread carbonaceous remains in the basal peat layers indicate more than just local disturbance of the vegetation. There is evidence from contemporary hunter–gatherer communities of the widespread use of fire to increase the stocking capacity of hunting grounds and to control herd movements[14], so that it is possible that sporadic burning was also carried out in the southern Pennine uplands before 3000 BC by Mesolithic hunters. Evidence of burning in the peats, however, continues upwards into the Sub-Boreal period, when Mesolithic populations had almost certainly been replaced by Neolithic cultures. Since at several sites in the southern Pennines there is apparent continuity of occupation from Mesolithic to Neolithic times[15], it is possible that the uplands continued to be used primarily as seasonal hunting grounds for many centuries after the first advent of Neolithic peoples (at the time of the Elm Decline). With the gradual development of more intensive agriculture, anthropogenic pressure on the uplands may ultimately have diminished, and with reduced frequencies of

burning, trees were able to colonise the drier shallower peats. The subsequent disappearance of these trees could have been the result of natural death from waterlogging consequent on peat accumulation, but since most tree remains in the peat are of relatively young trees it is more probable that deliberate woodland clearance was involved. Contrary to recent hypotheses[4] peat formation was already well advanced by this time.

J. H. TALLIS

Department of Botany,
University of Manchester,
Manchester M13 9PL, UK

1 Case, H. J., Dimbleby, G. W, Mitchell, G. F., Morrison, M. E. S, and Proudfoot, V. B., *J. R. Soc Antiq. Irl.*, 99, 39 (1969)
2 Goddard, A., thesis, Queen's Univ., Belfast (1971).
3 Mitchell, G. F., *Proc. 24th Int Geol Congr*, Symp. 1, 59 (Montreal 1972).
4 Moore, P. D, *Nature*, 241, 350 (1973).
5 Merryfield, D. L., and Moore, P. D., *Nature*, 250, 439 (1974)
6 Woodhead, T W., *J Linn. Soc. Bot.*, 37, 333 (1906).
7 Woodhead, T. W., *J Bot*, 62, 301 (1924).
8 Woodhead, T. W., *J. Ecol.*, 17, 1 (1929)
9 Conway, V. M., *J. Ecol.*, 34, 149 (1947).
10 Conway, V M., *J. Ecol.*, 42, 117 (1954).
11 Hicks, S P., *New Phytol*, 70, 647 (1971)
12 Birks, H. H., *Phil. Trans R Soc.*, B270, 181 (1975).
13 Radley, J, Tallis, J. H, and Switsur, V R., *Proc. Prehist. Soc.*, 40, 1 (1974)
14 Thomas, W. L. (ed.), *Man's Role in Changing the Face of the Earth*, 115 (University of Chicago, 1956).
15 Petch, J. A., *Early Man in the District of Huddersfield* (Tolson Memorial Museum, Huddersfield, 1924).

Regularities in duration of regional desert locust plagues

DURING a plague of desert locusts, *Schistocerca gregaria* (Forskal), extensive areas are infested by successive generations of gregarious populations, and for working purposes a plague can be considered to be in progress as long as the progeny of swarms remain in swarms. In the periods between plagues, or recessions, the species exists mainly in low density populations, and such small swarms as occasionally occur seldom persist beyond one generation.

The total area invaded by desert locust swarms during plagues extends from West and North Africa eastwards to Assam, and from the Middle East to Tanzania, and can be divided into four major regions—western, eastern, north-central and south-central[1] Each region contains complementary seasonal breeding areas connected by migration circuits which are characteristic, but not always closed. From records assembled at the Anti-Locust Research Centre (now Centre for Overseas Pest Research) it was possible to reconstruct the incidence of regional plagues back to 1900 in the north-central and south-central regions, and to 1860 in the western and eastern regions. The data obtained were examined to see if there was evidence of plague regularities or of periodicities such as have been suggested for the eastern[2] and western[3] regions. The main conclusions are summarised below; the full data and their discussion, and all the details of statistical analyses, are presented in refs 1 and 4.

Table 1 Frequency distribution of intervals between onsets of successive regional plagues

Interval (yr)	≤4	5–7	8–10	11–13	14–16	17–19
Frequency of occurrence	5	9	7	5	8	2

Parameters available for analyses were the lengths of regional plagues and recessions, and of the intervals between onsets of successive regional plagues. To have sufficient data for statistical tests, combined values from all regions were used in each analysis. This is not strictly valid, as swarms may move between regions, which are therefore not entirely independent. As the continuation of regional plagues seems to be independent of such move-

ments, however, we decided to pool the data and regard conclusions as tentative.

Table 1 shows that the intervals between onsets of successive plagues in the same region were very variable. The evidence of any regularity was not increased when plagues were considered in sequence, for in all the regions the serial correlation of successive intervals was not significant. As in three regions the correlations were negative, the intervals between onsets of one and the next but one plague were calculated, such double intervals were found, however, to vary from 9–28 yr. Thus the intervals provided no evidence of periodicity.

The frequency distributions of the durations of regional recessions and plagues (Fig. 1a and b) were considered separately Both were tested by examining their goodness of fit to the frequency distributions which could be expected on the following hypothesis : once started, a plague or a recession continues independently of how long it has lasted, until a chance occurrence of conditions which bring it to an end. The respective χ^2 tests showed that the actual distribution of recession lengths was consistent with this hypothesis, whereas the distribution of the plague lengths was not, so that for them the hypothesis could be reasonably discarded.

It follows that the termination of a plague may depend on how long it has already lasted. When estimates of the probabilities of a plague finishing in a particular year were calculated from the frequency distribution of plague lengths they suggested (Table 2) that the probability of a plague lasting only 1 yr was quite high, but that of it

Fig. 1 Frequency distribution of durations of regional desert locust recessions (a; $n = 36$) and plagues (b; $n = 40$).

finishing after 2 yr was very small, thereafter the longer the plague lasted, the more likely it was to end, with the probability of it lasting more than 8 yr being very small indeed. In effect, only one regional plague lasted more than 8 yr and in 55% of cases they lasted 6–8 yr (Fig. 1b).

The overall lack of periodicity in the onsets of regional plagues arises from a combination of the lengths of the plagues, which show some regularities, with those of recessions, which do not. The large proportion of brief regional recessions (Fig. 1a) may partly be caused by their termination by swarm invasions from outside. But some of them came to an end as a result of local plague upsurges, suggesting residual populations capable of a rapid rise to plague level.

Table 2 Estimated probabilities of a plague finishing in a particular year when it existed at the beginning of that year

Plague existing in year:	1	2	3	4	5	6	7	8
Probability of it finishing	0.18	0 00	0.06	0.10	0 18	0.36	0 47	0 88

From the frequency distribution of regional plague lengths it seems that plagues may be of two types—longer ones, which seem to carry the seeds of their own destruction, and short ones The termination of some recent plagues, both short and long[5], may have been contributed to by control measures. It is certain, however, that such measures used in earlier times could not have had any significant effect on swarming populations. It is also known that the ending of some of the plagues was associated with droughts[1,6]. It seems doubtful, however, that climatic factors, acting either directly or by differing from those to which locusts may have been preconditioned by the photoperiodic regimes of their progenitors[7], could have accounted for the termination of most of the longer plagues, which ended at comparatively regular intervals after their irregularly occurring onsets. A cumulative effect of parasites and predators could be consistent with such regular intervals, but a review of available data on effects of all biotic factors on this highly mobile species indicated that they probably did not materially affect large swarming populations[8].

The tendency of the longer plagues to last a comparatively regular and seldom exceeded period suggests that their declines may be brought about by some deterioration in populations persisting in a crowded state for a long succession of generations. Deleterious changes in high density insect populations are known[9-11] and in locusts the reproductive capacity becomes reduced on crowding[12]. This suggestion could be tested, in the event of another desert locust plague, by comparing in standard optimum conditions the survival and reproductive potentials of members of swarming field populations differing by known numbers of gregarious generations from the initial swarms.

Z. WALOFF
S. M. GREEN

Centre for Overseas Pest Research,
College House, Wrights Lane,
London W8 5SJ, UK

1 Waloff, Z , *Anti-Locust Mem.* (in the press)
2 Rao, Y. R., *The Desert Locust in India,* (Indian Council of Agricultural Research, New Delhi, 1960)
3 Pasquier, R , *Bull Soc Agric Algér* , 85 (no 509), 51–70 (1942).
4 Green, S M , *Anti-Locust Mem.* (in the press)
5 Rainey, R. C , *12th Int Congr Ent., London 1964,* 588 (1965)
6 Waloff, Z , *Anti-Locust Mem* , no. 8, 111 pp (1966)
7 Albrecht, F O , *Acrida,* 2, 97–107 (1973)
8 Greathead, D. J , *J. appl Ecol.,* 3, 239–250 (1966)
9 Wellington, W G., *Can. J Zool* , 38, 289–314 (1960).
10 Klomp, M., *A Rev Ent* , 9, 7–40 (1964)
11 Klomp, M., *Adv. ecol. Res.,* 3, 207–305 (1966)
12 Papillon, M , *Bull. biol. Fr. Belg.,* 102, 272–307 (1968).
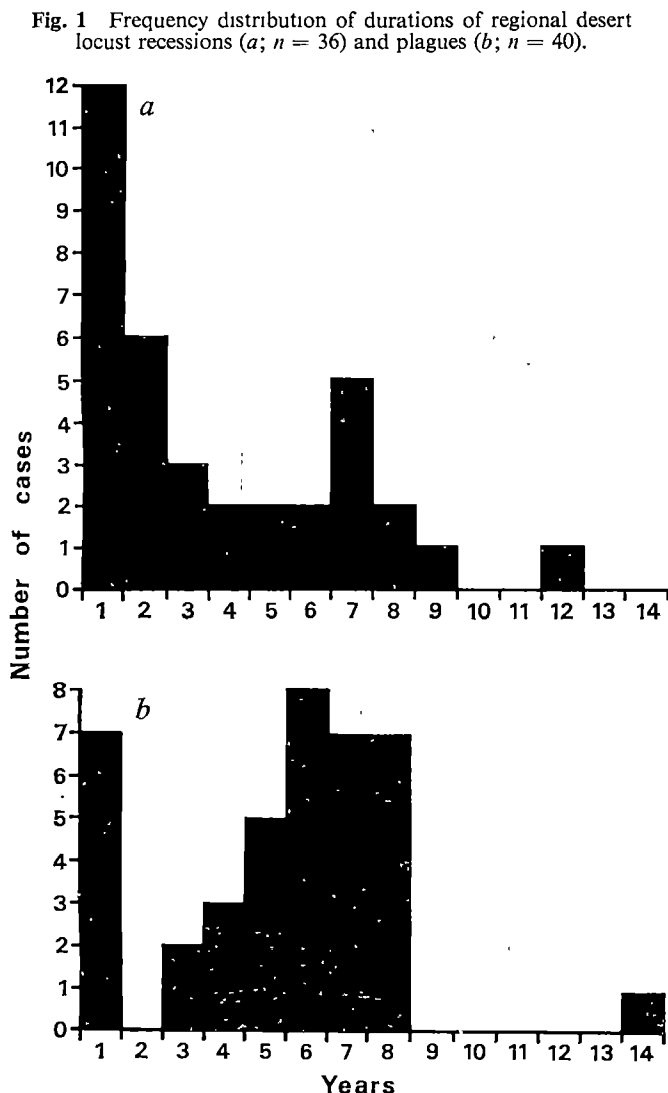
Development of a desert locust plague

THE role of phase change in the development of plagues of the desert locust, *Schistocerca gregaria* (Forskal), has been debated for many years[1-5]. Much confusion has arisen because changes in behaviour, form and colour, which have all been used as indicators of phase status, do not proceed at the same rates It is generally agreed, however, that behavioural changes are crucial in phase transformation. The subject at issue in the population dynamics of the desert locust is whether plagues, periods when extensive areas are infested by successive generations of gregarious populations, originate from an upsurge in numbers and associated transformation from the solitary-living to the gregarious state, or from successful breeding by gregarious populations postulated to survive continuously throughout the interplague recession periods[3]. Fundamental questions about the strategy of plague prevention depend on determining which of these hypotheses is correct[1,3,4,6].

An analysis of events preceding and during the 1968 desert locust plague has demonstrated the importance of phase transformation in plague development[6]. The upsurge of this plague, which began in mid-1967, was the result of an increase in numbers and densities of initially solitary-living locusts in successive generations. Each generation bred successfully following periods of rainfall over large parts of the normally arid recession area. It has been tentatively concluded from analysis of 36 occurrences of successful breeding that 25 mm is the threshold rainfall requirement for multiplication from mature parents to filial fledglings. This is slightly more than the 20 mm previously shown to be necessary for successful oviposition and incubation[7]. The timing of these falls of rain was such that a very short period usually elapsed between fledging and maturation, and this may be critical in upsurges.
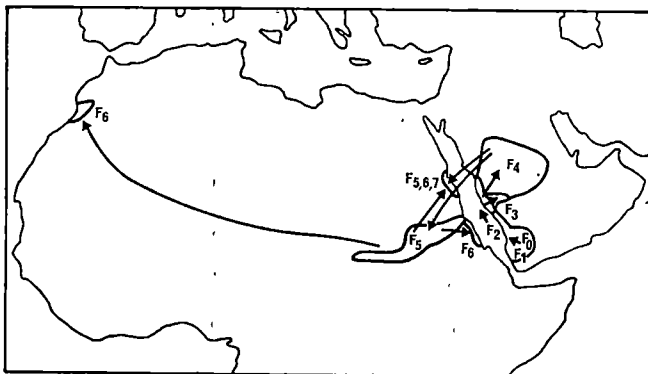
Fig. 1 A sequence of breeding by desert locusts. The first generation (F_0) bred in early 1967, and the F_8 generation in late 1968. There is no evidence of breeding by F_7 adults. Arrows indicate direction of displacement between generations. Breeding areas are not drawn to scale.

Small and usually transient swarming populations are intermittently reported during recessions[4,5]. Careful examination of all reports of desert locusts in 1966 and 1967 (ref. 6) suggests that the last two authentic swarms, before the buildup in mid-1967, were found in January 1966—one in Ethiopia and the other in Somalia. Neither produced gregariously-behaving adults in the next generation.

From mid-1967 to mid-1968 increasingly large numbers of locusts were reported from a number of places within the 14 million km² desert recession area stretching from West Africa to India. In each generation in each sequence of breeding there were progressive changes in the character of the populations, and the sizes of the infested areas. This can be illustrated by examining one such sequence, traced

over eight generations (Fig. 1). Detailed analysis has shown that the preliminary assessment of events outlined by Pedgley and Symmons[8] was substantially correct. It is almost certain that the first generation bred in the south-western interior of the Arabian peninsula in April–May 1967 (F_0). The second probably bred partly in that area and partly on the coastal plain of Yemen and Saudi Arabia in July 1967 (F_1). There is no evidence of gregarious behaviour in either of these generations, that is to say no bands of hoppers or swarms of adults were reported. One sequence that developed from this source involved a further generation breeding on the coastal plain after rain in November 1967, and two more on the coastal plain and in the interior of Saudi Arabia after rain in February and April 1968.
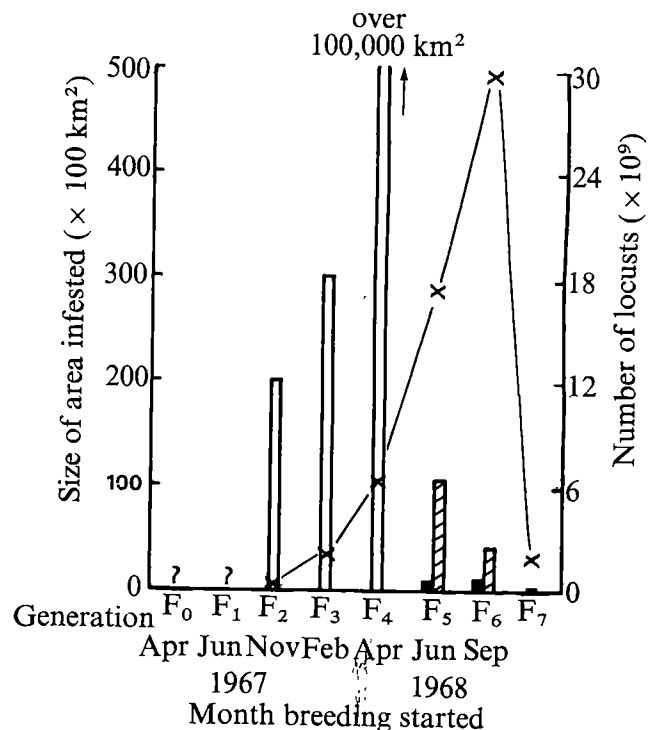
Fig. 2 The approximate numbers of locusts in each generation (×) and the gross areas infested by different types of population during the sequence of breeding shown in Fig. 1. Open bars show areas infested by locusts that were not fully gregariously-behaving; black, by swarms; striped, by bands. Gross infested area is the area needing treatment to reduce the population to an insignificant size.

In these three generations (F_2, F_3 and F_4) an increasingly large number of locusts were found in an expanding area (Fig. 2) and an increasingly large proportion of the population showed some gregarious behaviour. The F_5 generation, however, was the first in which almost all the locusts were in bands and swarms. From May–August swarms of this generation emigrated from Saudi Arabia. About half migrated south-west across the Red Sea to Egypt and Sudan, where they started to breed in June 1968. Once gregarisation had occurred the gross infested area was reduced by about an order of magnitude between generations F_4 and F_5 in this sequence, although the number of locusts continued to increase (Fig 2). Approximately half of the F_6 swarms emigrated from Sudan to Morocco, where low winter temperatures delayed maturation and almost all the swarms were killed before they could breed. Other swarms from Sudan migrated towards the Red Sea coastal plains; some were controlled and the remainder oviposited between September and November. There, the F_7 generation was much reduced by control, and the survivors died without producing gregarious locusts in the next generation. The progressive reduction in infested areas from F_5 to F_7 and in numbers from F_6 to F_7 (Fig. 2) resulted from control

Values of means of first canonical
variates of samples
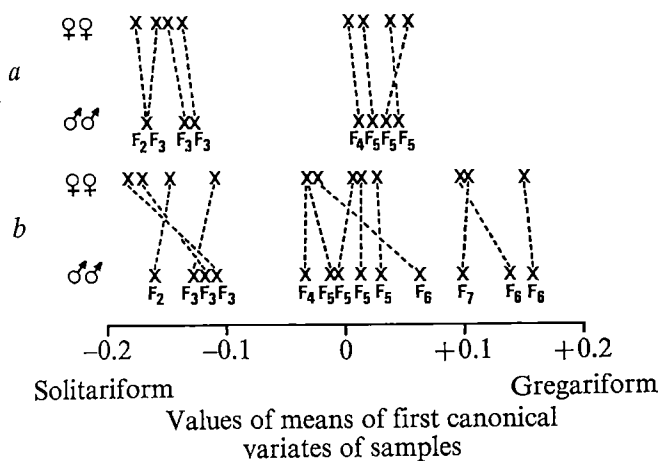
Fig. 3 Values of means of first canonical variates of samples from successive generations in two upsurge sequences. *a*, West Africa: F_2 Niger, September, 1967; F_3 Mali and Algeria, November 1967–March 1968; F_4 Mali, July 1968; F_5 Mali and Niger, September–November 1968. *b*, Sequence shown in Figs 1 and 2

measures and natural mortality, and cannot be taken to represent typical development during a plague.

Morphometrics are available for several samples from generations F_2 to F_7 in the above sequence The largest representative samples are shown in Fig. 3, which shows that the steady increase in gregarious behaviour was paralleled by a shift from the solitariform to the gregariform in the means of the first canonical variates[9]. The F_7 sample came from the Red Sea coast population that was the progeny of the most solitariform of the populations from which the F_6 samples were derived, so the shift towards gregariform continued in this generation The two more gregariform F_6 samples were derived from the swarms which moved westwards from Sudan towards Morocco. Similar shifts can be seen in other sequences of breeding that contributed to the plague, the most complete of which is also shown in Fig. 3

Phase transformation was progressive, and an integral part of the development of the most recent desert locust plague. One aspect of phase transformation is that during swarm formation (Fig. 2), the gross area requiring treatment contracts. The same area dosage rates are required for effective control of locusts whatever their density, so it is clearly more economical to concentrate control on the initial gregarious populations than to attempt to control the much larger areas infested at earlier stages in an upsurge, or the subsequent swarming generations which may be larger. A fuller account of this work will be published elsewhere.

L. V. BENNETT

Centre for Overseas Pest Research,
College House, Wrights Lane,
London W8 5SJ, UK

1 Gunn, D. L , *A. Rev. Ent.*, 5, 279–300 (1960)
2 Kennedy, J. S., *Colloques int. Cent natn. Rech scient* , No 114, 283–297 (1962).
3 Rainey, R. C., *13th Int Congr Ent., Moscow, 1968*, 2, 379 (1971)
4 Roffey, J., Popov, G , and Hemming, C F , *Bull ent. Res* , 59, 675–680 (1970)
5 Waloff, Z , *Anti-Locust Mem.*, No. 9 (1966)
6 Bennett, L. V., thesis, Univ London (1974).
7 Magor, J , thesis, Univ Edinburgh (1962)
8 Pedgley, D , and Symmons, P. M , *Weather*, 23, 484–492 (1968).
9 Symmons, P. M , *Bull ent Res* , 58, 803–809 (1969).

Seed-borne microorganisms
stimulate seedcorn maggot egg laying

LARVAE of the seedcorn maggot, *Hylemya platura* (Meigen) may damage or kill young plants of many crop species by feeding on the cotyledons and plumules (Fig. 1). Damage may

be caused by larvae already present in the soil before the seeds are planted[1] or by larvae that hatch from eggs laid near germinating seeds[2,3]. It has been assumed that the stimulus for oviposition is provided by the germinating seeds themselves but we suspected that seed-borne microorganisms growing on seed exudates could produce metabolites that promote oviposition. Many metabolic processes begin at the onset of seed germination, and concurrently organic substances are leached from the seeds which provide substrates for microorganisms. We now report that microorganisms growing on substrates from seeds produce metabolites which stimulate egg laying in *H. platura*, and that germinating microorganism-free seeds are not stimulatory. This is an excellent example of a complex ecological interaction between an insect pest, the host plant and organisms of the microenvironment.
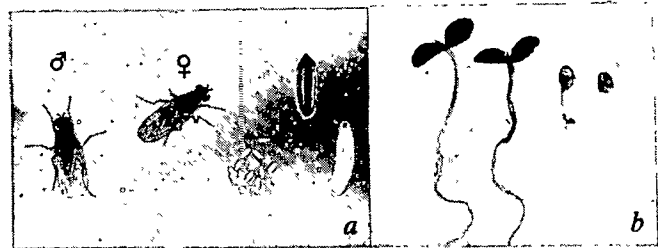
Fig. 1 *a*, Flies, eggs, puparium and larva of *H. platura*. *b*, Examples of varying degrees of injury on squash seedlings caused by earlier maggot feeding.

Squash (*Cucurbita pepo L.*) seeds, which elicit profuse oviposition[3] were planted in a system designed to prevent microbial contamination and to provide a suitable ovipositional substrate. Seeds or their microorganisms were incubated for 24 h for complete seed imbibition and/or microbial growth to occur. Flies that had been reared using a modified Harris technique[4] were then given access to beakers containing either the seeds or microorganisms for 24 h, immediately after which the eggs were counted[5]. All experiments were repeated five times.

We compared microorganism-infested seeds with seeds free from infestation. Earlier work demonstrated that Butternut squash seeds were preferred by the flies[3] but we could not eliminate seed-borne microflora from this variety without killing the seeds. We therefore included Table Queen seeds whose microflora could be eliminated without imparing germination[6]. With both varieties, most egg laying occurred in beakers containing seeds infested with microorganisms,

Table 1 Oviposition by *H. platura* in beakers containing squash seeds incubated on water agar and covered by a layer of sterile acid-washed quartz sand

Seed	% Total eggs oviposited
Infested Butternut	47
Infested Table Queen	27
No seeds	17
Microorganism-free Table Queen	8

The results are all significantly different at the 5% level as determined by Duncan's multiple range test on arcsin transformations.

significantly fewer eggs were found in containers with no seeds, and very few with microbe-free seeds (Table 1). These results show that microorganisms on squash seeds stimulate *H. platura* oviposition. In addition, germinating squash seeds in the absence of microorganisms may deter oviposition (Table 1). Two additional tests not reported here gave similar results.

Before attempting to identify some of the microorganisms involved we had to develop a system enabling us to isolate them in pure culture. Since either germinating seeds or soil may provide the substrates for growth of the stimulatory microorganisms, we developed a system using squash seed exudate

Table 2 *H. platura* oviposition in beakers containing various media and microorganisms from squash seeds

Medium	% Total eggs oviposited*
SSEA†+microbes	49.8 (a)
SEA‡+microbes	18.5 (b)
SEA	14.5 (b)
SSEA	8.9 (b)
Water agar	8.4 (b)

The microorganism inoculum was a mixture of microorganisms obtained from the Butternut seeds. The substrate was in polypropylene reservoirs surrounded by water agar and covered by a sterile glass-fibre filter and a layer of sterile acid-washed quartz sand.

*Percentages followed by different letters are significantly different at the 5% level as determined by Duncan's multiple range test on arcsin transformations.

†Squash seed exudate agar (SSEA) was prepared from a mixture of 100 g of Butternut squash seeds in 1 l of distilled water that had been shaken for 16 h. The seeds were removed by filtering through cheesecloth, the liquid portion clarified by centrifuging at 10,000g for 10 min followed by filtration through a Reeve-Angel 934 H glass filter. The resulting liquid was heated with sufficient agar to make a 2% solution and sterilised by heating at 120 °C for 20 min.

‡Soil extract was prepared by heating a mixture of 100 g of greenhouse soil with 1 l of tapwater at 100 °C for 30 min, then adding about 1 g of $CaCO_3$, filtering, and finally sterilising in the autoclave for 20 min at 120 °C. The complete soil extract agar (SEA) was prepared by mixing 100 g of soil extract, 0.5 g K_2HPO_4, 1 g of glucose, 20 g of Bactoagar and 1 l of water. This mixture was heated until the agar was in solution and then sterilised at 120 °C for 20 min. The pH of SSEA was 4.9–5.1, that of SEA was adjusted to 8.4.

agar (SSEA) or soil extract agar (SEA) (Fig. 3). The media were inoculated with 200 µl of a mixture of microorganisms obtained by shaking 10 g of the highly stimulatory Butternut seeds for 16 h in 100 ml of distilled water at 23–25 °C; dilution plates of the mixture yielded 10^{10}–10^{12} microorganisms ml^{-1} with Bacto-Nutrient Agar. When inoculated, heavy microbial growth occurred on both SSEA and SEA but the former was preferred for oviposition (Table 2). These results indicate that squash seed exudates provide substrates for microorganisms which produce stimulatory metabolites and provide a system for routine assay.

Table 3 Oviposition by *H. platura* in beakers containing various microorganisms grown on SSEA

Microorganism	% Total eggs oviposited*
Pseudomonas+Torulopsis aeria	47 (a)
Pseudomonas sp	26 (b)
Torulopsis aeria	22 (b)
None	6 (c)

The substrate was in a polypropylene reservoir surrounded by water agar and covered by a sterile glass-fibre filter and a layer of sterile acid-washed quartz sand.

*Percentages followed by different letters are significantly different at the 5% level as determined by Duncan's multiple range test on arcsin transformations.

Among the pure cultures obtained from Butternut seeds, a bacterium, a *Pseudomonas* sp. (Migula)[7] and a yeast, *Torulopsis aeria* (Saito) Lodder[8], were the most effective elicitors of oviposition. Isolates of these organisms have been deposited in the USDA Northern Marketing Research Division Collection, Peoria, Illinois, and have been designated NRRL B-4287 and NRRL Y-7845, respectively. Mixed cultures of these two organisms stimulated oviposition to a greater extent (Table 3).

This work provides a model system for metabolite identification. Syntheses of ovipositional stimulants may improve population monitoring of *H. platura* in the field as is the case with pest monitoring and modelling using synthetic insect sex pheromones[9].

We thank Glenda Nash and Helen Van Buskirk for technical assistance.

C. J. ECKENRODE
G. E. HARMAN
D. R. WEBB

New York State Agricultural Experiment Station,
Cornell University,
Geneva, New York 14456

[1] Miller, L. A., and McClanahan, R. J, *Can. Ent* **92**, 210–221 (1960)
[2] Barlow, C. A., *Ent exp. appl.*, **8**, 83–95 (1965).
[3] Yu, C. C., Webb, D R., Kuhr, R J., and Eckenrode, C. J., *Environ. Ent* (in the press).
[4] Harris, C R., Svec, H. J., and Begg, J A., *J. econ Ent.*, **59**, 407–410 (1966).
[5] Horsfall, W. R, *Mosquito News*, **16**, 66–71 (1956).
[6] Harman, G E., and Pfleger, F. L., *Phytopathology*, **64**, 1339–1344 (1974).
[7] Buchanan, R. E, and Gibbons, N. E., *Bergy's Manual of Determinative Bacteriology*, ninth ed. (Williams and Wilkins, Baltimore, 1974).
[8] Lodder, J., and Kreger-Van Rij, N. J. W., *The Yeasts—A Taxonomic Study* (Interscience, New York, 1952).
[9] Tette, J. P., in *Pheromones* (edit. by Birch, M. C.), 399–410 (American Elsevier, New York, 1974)

Defensive stoning by baboons

REPORTS of the use of tools in offence or defence by wild animals is limited to accounts of chimpanzees throwing branches at conspecifics, potential predators and at human observers[1]. Anecdotal accounts of stone throwing by baboons[2] have been dismissed on the basis of the unreliability of correspondents and the improbability of oriented throwing by a quadruped anatomically incapable of overhand throwing[3]. In spite of several years of field study elsewhere in Africa, often in rocky terrain, there are no reports by professional field observers of deliberate stone throwing by baboons[4–7].

Nevertheless, in the course of a one-year study of three chacma baboon (*Papio ursinus*) troops living on the desert floor of the Kuiseb Canyon in South West Africa we observed numerous instances of stone release directed towards us.

Stoning by these baboons is done from the rocky walls of the canyon where they sleep and retreat when they are threatened by real or imagined predators. Stones are lifted with one hand and dropped over the side. The stone tumbles down the side of the cliff or falls directly to the canyon floor. We recorded the details of 23 such incidents involving the voluntary release of 124 stones towards us. All age and sex classes larger than 2-yr-olds participated at least occasionally in stoning (Table 1). But the most frequent participants are 4-yr-old and older males. The unhabituated nature of the troop members and the early stage of the study made individual identification impossible.

These stoning activities were accompanied by escape movements and typical "wahoo" alarm and barking calls, further identifying the context of the event as a predator–prey interaction. Such vocalisations are given typically in the presence of other predators such as leopards and sometimes hyaenas. The only animals preying on baboons in the Kuiseb Canyon today are the spotted hyaena (*Crocuta crocuta*), the brown hyaena (*Hyaena brunnea*) and man. Leopards lived in the canyon until recently but are now absent. Baboons have probably not been hunted by man in this canyon in recent years.

Stoning is aimed in the sense that the stone is released in such a way that it falls towards the observer, the stoning individual having moved to a position on the cliff directly above or opposite the observer. A stone is picked up, sometimes by prying it loose from the substrate, and released with an underhanded shovelling motion, or is simply dropped over the edge. This frequently resulted in stones whizzing over our heads. Usually we could dodge; but occasionally two or more individuals release stones at approximately the same time, complicating evasion. Stoning persisted when we were far enough out from the walls to preclude any possibility we might be struck. On one occasion we saw a stone released in the typical manner when we were above the releasing individual.

Stoning was never observed in the context of intraspecific

encounters, either in intratroop combat or in intertroop encounters. Baboons remove stones from sleeping ledges when these sites are occupied at dusk. The material removed at such times is copious and gives the opportunity to evaluate the selectivity of defensive stoning. Of 200 randomly selected stones picked up from below a sleeping cliff following occupation of that cliff by one troop the mean size was 9 cm × 5 cm and weighed 88 g. The mean size of 22 stones released towards us from that cliff was 165 cm × 104 cm and weighed 583 g. Evidently baboons select relatively large stones to release towards intruders.

A special set of conditions is required to elicit this stoning behaviour. The initial response by baboons to our presence in the Kuiseb Canyon was to flee up the rocky slopes of the canyon wall. No stones are released in such circumstances, although occasionally one may be kicked free and tumble down the cliff. Late in the year of our study the troops became more accustomed to us and, although they retreated from our close approach, they dropped no stones. Stoning occurred only when a troop was partially habituated to our presence. This occurred in the middle months of our study and when we returned following a 2-month absence.

Table 1 Age/sex distribution of stoning individuals in three troops

Age/sex (No. of individuals, 3 troops)	No. of incidents	Expected incidents
I (11)	0	2.6
J1, J2 (10)	0	2.4
J3 (11)	5	2.6
J4M (2)	2	0.5
AF (21)	2	5.0
SAM (7)	3	1.7
AM (17)	7	4.1
Unknown	4	—

Expected values are derived from the age/sex distribution of the 79 individuals comprising the three troops observed.
I, Infant; J, juvenile; AF, adult female; SAM, subadult female; AM adult female.

For stoning to take place there must be associated cliffs with free or loose rocky material. Individuals initiating stoning generally persist with several stones. If loose material is exhausted the thrower may work vigorously, attempting to free loose rocky material from the canyon walls.

Stones were never seized when the relevant conditions prevailed and the troop was on a talus slope rather than on a steep cliff. The threat must come from beneath the cliff to elicit stoning. During some of our later observations of this behaviour we deliberately elicited stoning either to gain additional data or to demonstrate it to other observers. By carefully gauging the movement of the troop we were able to approach them when they were near a suitable cliff. Our presence moved them up on to it. By remaining beneath their cliff but out of range we were regularly able to elicit stoning behaviour. The need for troops to be partially habituated to begin stoning may explain the confusion concerning the reality of aimed stone throwing by baboons elsewhere in Africa. If stoning does occur elsewhere in Africa it could easily be missed. Animals habituated away from cliffs may not throw stones when observations are made in an otherwise appropriate environment.

WILLIAM J. HAMILTON III
RUTH E. BUSKIRK
WILLIAM H. BUSKIRK

Division of Environmental Studies,
University of California,
Davis, California 95616

1 Goodall, J., *Nature*, 201 1264 (1964)
2 Kortlandt, A , and Kooij, M , *Symp zool Soc., Lond*, 10, 61 (1963).
3 Washburn, S L., and Jay, D. C., *Curr Anthrop.*, 8, 253 (1967).
4 DeVore, I , and Hall, K R. L , in *Primate Behaviour* (edit by DeVore, I.), 20 (Holt, London, 1965).

5 Kummer, H , *Social Organisation of Hamadryas Baboons* (University of Chicago Press, Chicago, 1968).
6 Stoltz, L. P , and Saayman, G. S., *Ann. Trans Mus*, 26, 99 (1970).
7 Altmann, S. A , and Altmann, J , *Baboon Ecology* (Karger, Basel, 1970)

Eccentricity-specific dissociation of visual functions in patients with lesions of the central visual pathways

THE human retina projects not only to the lateral geniculate bodies and from there to the visual cortices, but in a parallel fashion to several other subcortical centres as well. One such centre is the superior colliculus, and it has been suggested that a rudimentary discrimination capacity of the locus of visual stimuli may be mediated by this structure[1]. Results of anatomical studies with subhuman primates indicate that the macular region does not project to the superior colliculus[2,3] (or that it is represented by a different population of retinal fibres which have not yet been identified by anatomical techniques[4]). The macular region seems to project only to the lateral geniculate bodies and from there to visual cortices, whereas the retinal periphery projects to several subcortical centres. This raises the question of whether a similar dissociation of projection between the central and peripheral retina also can be found in the human visual system. We present data obtained from patients with lesions of the central visual pathways which indeed suggest such a dissociation.

Besides kinetic and static perimetry[5], the measurement of the critical flicker fusion (CFF) has proved to be a fruitful technique in the evaluation of visual dysfunctions in patients. A cortical lesion seems to result almost always in a diminution of the CFF in the contralateral visual field[6,7], also affecting the intact regions of the visual field and even the ipsilateral half field[8]; it has been suggested that the diminution of CFF in cases of brain injury is associated with changes in other visual functions such as the increment threshold[9], by which retinal sensitivity is measured, although this claim does not seem fully supported by the published material.

Table 1 Effect of stimulus eccentricity on CFF (Hz)

Patient	Contralateral to				Δ5°	Δ20°
	Lesion side		Intact side			
	20°	5°	5°	20°		
E A.	23.7	19.5	30.6	25.3	—11.1	—1.6
F.H.	31.0	26.0	33.3	29.0	—7.3	+2.0
G.J	30.4	30.5	31.6	30.3	*—1.1	+0.1
	Left side		Right side			
Control subjects (n=9)	30 8 (23 5–39.1)	32.1 (25 5–36.9)	32.0 (27.6–36.9)	30.9 (23.0–40.8)	0.1	0.1

$*P \leqslant 0.01$

We measured the CFF in three patients with injuries of the central visual pathways and in nine control subjects. Two patients (see Table 1, E. A. and F. H.) had homonymous upper quadrant defects, whereas one patient (G. J.) had no obvious visual field defects, but had received a penetrating brain injury by a gunshot that ended in the lateral and caudal thalamic nuclei presumably injuring fibres of the geniculo-cortical pathway. The CFF in the patients and control subjects was measured at 5° and 20° eccentricity along the 225° (left lower quadrant) and 315° (right lower quadrant) meridians. These eccentricities were chosen because the anatomical evidence from primates suggests that the borderline of dissociation of central pro-

jection is beyond 5° but below 20° eccentricity[3]. For stimulus presentation the Tubinger perimeter[5] was used. The CFF was measured binocularly after a careful monocular perimetry had been carried out. The diameter of the stimulus was 116 arc min visual angle, its luminance and the background luminance were 10 apostilb (3,183 cd m^{-2}). The red fixation point had a diameter of 30 arc min visual angle. The fixation was observed by the experimenter throughout the CFF measurements. For determination of the CFF, the ascending method of limits was used, starting at a frequency well below the fusion threshold. With the exception of one patient (G.J.) seven measurements were taken at each eccentricity. The means of these measurements are shown in Table 1 for the patients E.A. and F.H. and in averaged form for the nine control subjects (the range is given in parentheses). The measurements on patient G.J. were much more extensive. On 7 different days 15 CFF values for 5° and 10 CFF values for 20° eccentricity were determined, each value being the average of 5 single measurements. The mean values of these 10 and 15 values are also shown in Table 1.
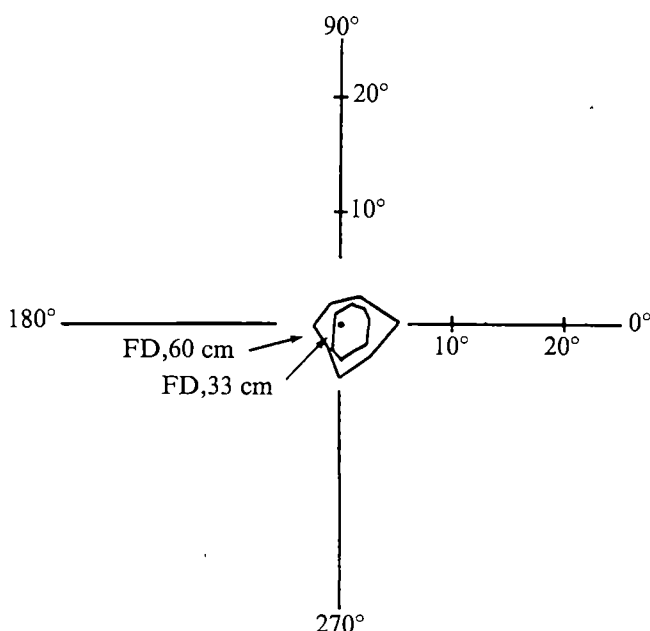
Fig. 1 Peephole visual field of a patient (H. H.) with a vascular lesion of both occipital lobes. S, Visual stimulus used to measure the visual field (116 arc min visual angle, 1,000 apostilb), BG, light intensity of the homogeneous background (10 apostilb); FP, fixation point (60 arc min visual angle, 1,000 apostilb); FD fixation distance.

The results (Table 1) show no difference of CFF for the control subjects if the measurements of 5° and 20° on both sides of the visual field are compared. As would be expected for these conditions, CFF is slightly lower towards the periphery[10]. For the patients E.A. and F.H. we observe a striking diminution of CFF contralateral to the lesion side for 5° eccentricity; CFF is 11.1 and 7.3 Hz less than contralateral to the intact side. Such a diminution of CFF is not seen for 20° eccentricity. For patient G.J. we observe no difference of CFF, if the measurements at 20° eccentricity on both sides of the visual field are compared (Wilcoxon test: $T=12$, $P \geqslant 0.05$). Although there is only a 1.1 Hz difference in CFF if the measurements at 5° eccentricity are compared, this difference turns out to be highly significant (Wilcoxon test: $T=1$, $P \ll 0.01$).

Our observations can be summarised as follows : a lesion of the central visual pathways results selectively in a diminution of CFF in the perifoveal area leaving CFF in the periphery of the visual field apparently unaltered. The diminution of CFF is observed in the intact part of the

visual field, and it may even be observed if no obvious visual field defect is present (patient G.J.) stressing the sensitivity of CFF for diagnostic purposes.

We suggest that the selective influence of a lesion of the central visual pathways on CFF in the perifoveal area but not in the periphery of the visual field is a consequence of a dissociation of retinal projection as it has been observed in lower primates. This is supported by a further observation in one patient (G.J.). Latencies of saccadic eye movements are much longer for targets at 5° eccentricity when they lie contralateral to the lesion side, but such a difference was not seen for targets with 20° eccentricity. An eccentricity-specific dissociation was not seen for increment threshold, however, which was higher at all eccentricities between 5° and 25° contralateral to the lesion side. The observation of a dissociation seems to be dependent on the selection of the test. Furthermore, the diminution of CFF at 5°, but not at 20°, suggests that the retino-collicular and cortico-collicular pathways are somehow involved in the perception of flicker. In experiments on programming of saccadic eye movements[11] it has been observed that targets within 10° of the visual axis are reached by one eye movement, whereas targets beyond 10° elicit at first an orienting response bringing the line of sight closer to the target, and then in a second step the target is reached by a corrective saccadic eye movement. These observations and the facts presented here favour the hypothesis that visual information is analysed in a qualitatively different manner by the nervous system according to its distance from the visual axis. The anatomical observations in subhuman primates would suggest that this differential analysis is dependent on a dissociation of the retinal projection in the human visual system.

ERNST PÖPPEL
DETLEV VON CRAMON
HERBERT BACKMUND

Max-Planck-Institut für Psychiatrie,
8 München 40,
Kraepelinstrasse 10, Germany

1 Poppel, E., Held, R., and Frost, D, *Nature*, 243, 295 (1973).
2 Brouwer, B., and Zeeman, W. P. C., *Brain*, 49, 1 (1926).
3 Wilson, M. E., and Toyne, M. J., *Brain Res.*, 24, 395 (1970).
4 Schiller, P. H., Stryker, M., Cynader, M., and Berman, N., *J. Neurophysiol.*, 37, 181 (1974)
5 Sloan, L. L., *Archs Ophthalmol*, 86, 612 (1971).
6 Hylkema, B S., *v. Graefes Arch Ophthalmol*, 146, 110 (1944).
7 Teuber, H -L., Battersoy, W. S., and Bender, M B., *Visual Field Defects after Penetrating Missile Wounds of the Brain* (Harvard University Press, Cambridge, Massachusetts, 1960)
8 Goldman, P. S., Lodge, A., Hammer, L R., Semmes, J., and Mishkin, M., *Neuropsychol* 6, 355 (1968).
9 Koerner, F., and Teuber, H -L, *Expl Brain Res*, 18, 88 (1973).
10 Brown, J. L, in *Vision and Visual Perception* (edit. by Graham, C H.), 251 (Wiley, New York, 1965)
11 Frost, D., and Poppel, E., *Pflugers Arch* (*Eur J Physiol.*), R64 (1974)

Evidence for visual function mediated by anomalous projection in goldfish

IN goldfish, there is a complete decussation of the optic nerves and only contralateral projection to the optic tectum is present. If one tectum is removed surgically, a few months later, the contralateral eye projects to the remaining ipsilateral tectum, superimposes the normal projection in a mirror-image fashion and retains its normal polarity[1]. The method of mapping visuo-tectal projection involved presynaptic recordings of the optic fibre terminals in the tectum. It is not known, however, whether or not the anomalous projection is able to mediate visual behaviour. To determine whether the regenerated optic nerve fibres have formed functional connections in the tectum, post-synaptic recordings and (or) behavioural tests are required. We report here a preliminary finding that the anomalous ipsilateral

visuotectal projection, after removal of the contralateral tectum, may mediate the detection of a visual stimulus, with a threshold not significantly different from the normal projection. Sharma[1] has observed that in animals with complete removal of the right tectum "after removal of the right eye in these animals, the behaviour mediated by the left eye was apparently normal", and here we supplement those observations with psychophysical data.

Three adult fish were prepared as described previously[1]; the entire left optic tectum was removed by cutting and suction, and the skull flap replaced; both eyes were left intact. Psychophysical testing began 125 d after tectal removal, at which time an organised ipsilateral projection is present. The fish was restrained in a transparent plastic envelope, and respiration recorded by suspending a thermistor, operated in its heating mode, in front of the fish's mouth. Breathing produced a temperature change in the thermistor; the resistance changes were recorded on a polygraph and were also converted to digital form with a saturating amplifier and sensitive relay. Details of the method are described elsewhere[2]. The fish was suspended in an experimental tank with one eye facing a stimulus field (4×6 cm) at a distance of 45 cm. The field was illuminated constantly at a luminance of 3.3 cd m^{-2}. The stimulus consisted of five vertical bright bars, 15 mm across, which were moved back and forth twice each second at a rate of 11 cm s^{-1}.

Carbon rod electrodes were suspended on either side of the fish, through which an AC shock could be delivered; shock intensity was adjusted to the minimum level which would reliably produce a deceleration of respiration. Visual conditioned responses were established by the following procedure: (1) there was a 10 s period in which the number of breaths was counted and a stimulus was not presented; (2) this was followed by a 20-s delay; (3) a stimulus was turned on for 10 s and the number of breaths counted; (4) a shock of 250 ms duration was delivered on every trial when the stimulus was turned off. The criterion for conditioning (and detection of the stimulus) was as follows: total number of breaths during the 10-s stimulus was two less than during a similar period without a stimulus, and(or) there was at least one inter-breath interval greater than 1 s during the stimulus. To check for false-positive responses, a series of trials was run without a stimulus being presented during the second 10-s period but with electric shock being delivered as on stimulus trials; the incidence of response by the criterion of two-fewer breaths was less than 2%, and an inter-breath interval of more than 1 s was never recorded. Trials were presented randomly in time with an average interval of 3 min, to prevent temporal conditioning.

The number of breaths per 10-s interval varied between subjects and from day to day within a single subject, in the range 13–22. As an example of consistency of response, the mean breath rate for 1 d was 16.75 per 10 s (s.e. 0.38).

After reliable conditioned responses were established (which usually required only 8–10 trials), thresholds were determined by a staircase method: if a conditioned response was obtained to a given stimulus, the next stimulus was reduced in luminance by 0.3 log unit; if a response was not obtained, the next stimulus was increased by 0.3 log unit. This procedure continued until the reversals in the staircase reliably occurred in a small range of luminances.

All three fish produced vigorous conditioned responses with either eye facing the stimulus field. There was no reliable difference between the thresholds obtained with the two eyes. All measurements were made at least four times. The fact that thresholds were nearly identical with the two eyes argues against the possibility that responses with the right eye facing the stimulus were mediated by scattered and reflected light in the tank, which stimulated the left eye. This possibility was completely eliminated by removing the left eye, under anaesthesia, of one fish: there was no measurable change in threshold with the right eye facing the stimulus.

The visuotectal projection was mapped 148 d after tectal removal, and 4 d after the left eye was removed. The remaining (right) eye projected on to the remaining ipsilateral (right) tectum in normal retinotopic order, as described previously[1]. The anomalous ipsilateral visuotectal projection in goldfish is then able to mediate visual behaviour with sensitivity as great as the normal projection. The conditioned responses cannot be mediated by the remaining normal retinothalamic projection, as tectal lesions result in absolute scotomas[3], although opto-kinetic nystagmus is still present[4]. Experiments are in progress to investigate visual acuity, summation area and visual direction in the reinnervated tectum.

DEAN YAGER

State College of Optometery,
State University of New York,
131 East 23rd Street,
New York, New York 10010

S. C. SHARMA

Department of Ophthalmology,
New York Medical College,
1249 Fifth Avenue,
New York, New York 10029

1 Sharma, S C, Expl Neurol, 41, 661–669 (1973).
2 Northmore, D., and Yager, D., in Vision in Fishes (edit by Ali, M. A), 689–704 (Plenum, New York, 1975).
3 Schwassman, H. O , and Krag, M. H., Vision Res., 10, 29–42 (1970).
4 Easter, S. S., Landreth, G. E., and Northcutt, G, Soc. Neurosci. Abstr., 4, 197 (1974)

Thymus rudiment of the athymic nude mouse

IT is accepted that the polycystic organ, composed of branching ducts and differentiating during the first month of postnatal life into a system of interconnected cysts and acinar arrangements of serous and mucoid cells and containing almost no lymphoid cells, represents the dysgenetic thymus of the nude homozygote mouse[1,2]. No epithelial cystic cells and no cells with specific inclusions which may be the cellular source of the thymic factors affecting pathways for differentiation of T lymphocytes, were found in the polycystic organ[3,4]. The nude mouse has, however, not only the prethymic cells capable of differentiating into T cells in a thymic graft from an euthymic donor[5], but also surprising numbers of θ-positive cells in the spleen[6]. It is suggested that these T cells may leak across the placenta from the euthymic mother into the nude foetus, may differentiate under the influence of the maternal thymic hormone, or may acquire their T-cell properties by some kind of non-thymic induction[7].

Fig. 1 The lymphoepithelial rudiment of a nu/nu (BALB/c) male aged 122 d. H & E, ×160.
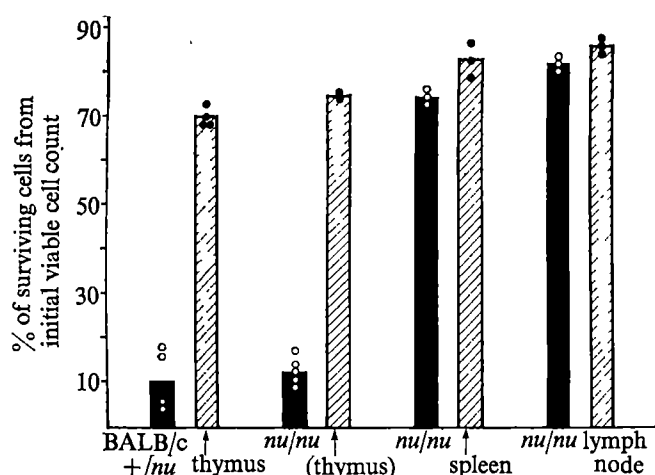
Fig. 2 Sensitivity of cells from euthymic mouse thymus and *nu/nu* lymphoepithelial rudiment (thymus?), spleen and lymph nodes to anti-θ antibodies. Solid columns, anti-θ-C3H+ complement; hatched columns, C only.

We have made histological serial sections of the entire area from the thyroid cartillage caudally to the bifurcation of the trachea in 42 *nu/nu* mice obtained from cross–intercross breeding of CBA mice with *nu/nu* mice, in 8 *nu/nu* mice from the eighth backcross to BALB/c mice (donated by Dr C. W. Friis) and in 3 *nu/nu* mice from the third backcross to C3H mice. All animals (both sexes, aged 30–122 d) were from *nu/nu* male × hybrid female matings. Between five hundred and two thousand haematoxylin–eosin stained sections of each mouse have been studied.

A paired ovoid structure (transversal sections usually not exceeding 400×200 μm in diameter), composed of condensed lymphatic tissue and situated ventrally to the trachea and laterally to the polycystic organ was found in all *nu/nu* mice (Fig. 1). The structure was surrounded by a thin fibrous capsule and a poorly demarcated sinusoidal space underneath the capsule. On the medial aspect it was bordered with mature fat tissue. Typical small lymph nodes were found in the same sections laterally or dorsally to the trachea. In one *nu/nu* (CBA) male aged 4 months a single ovoid thymus-like organ with a dense cortical and loose medullary component and more pronounced subcapsular sinus was found in the same location as the lymphatic tissue described above.

Fig. 3 The lymphoepithelial rudiment of a *nu/nu* (C3H) female aged 88 d. Lymphocytes in the cortical part surrounding epithelial cells (arrow). Toluidine blue, ×1,520.
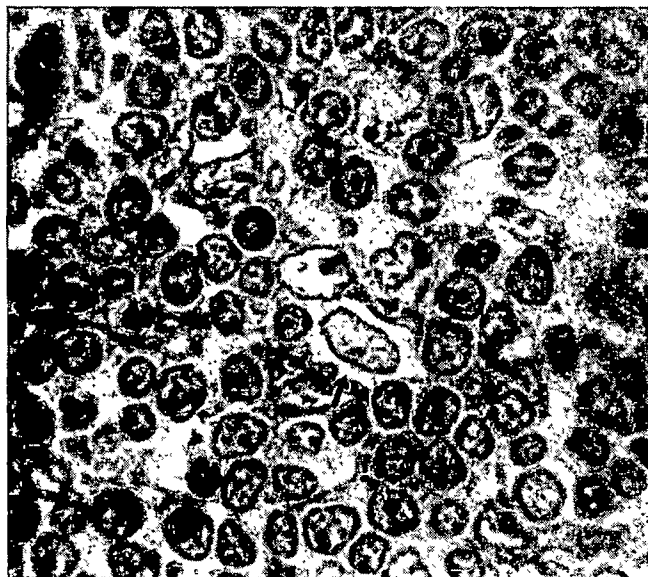
This lymphatic rudiment was isolated from 12 *nu/nu* mice of all three genetic backgrounds and processed for electron microscopy (glutaraldehyde fixation, Vestopal embedding, semi-thin sections stained with toluidine blue, ultrathin sections contrasted with uranyl acetate and lead citrate observed in a Tesla BS 242 D electron microscope). Lymphoid cells teased from the rudiment of five *nu/nu* (BALB/c) mice were assayed with anti-θ C3H antibodies[8]. These were obtained from the ascitic fluid of AKR mice immunised repeatedly with C3H thymocytes and injected with S37 cells 14 d before killing. The *nu/nu* lymphoid cells were incubated with anti-θ C3H in Eagle's MEM for 30 min at 0 °C and subsequently with guinea pig complement (1:10) for 45 min at 37 °C. $2 \times 10^5 - 4 \times 10^5$ cells from the rudiment were used and compared in the anti-θ assay with *nu/nu* spleen and lymph node cells and with BALB/c thymic cells. Control samples were incubated only in MEM and complement (30 min and 45 min). Each suspension was counted in a Burker chamber and in the Trypan-blue exclusion test before and after incubation and the results expressed as a percentage of viable cells from the initial count.
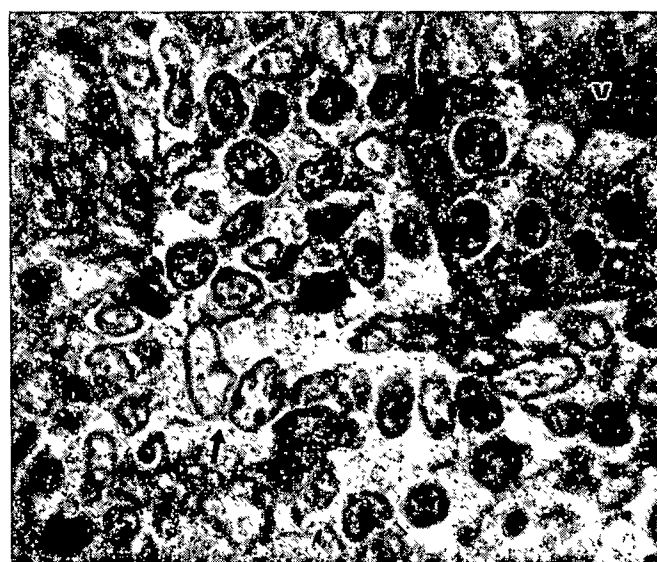
Fig. 4 The lymphoepithelial rudiment of a *nu/nu* (C3H) female aged 88 d. Lymphocytes and epithelial cells (arrow) in the central portion A high endothelium venule (v) with lymphocytes in passage through the wall. Toluidine blue, ×1,400.

Whereas few *nu/nu* spleen cells and even fewer lymph node cells were killed by anti-θ antibody and complement, the proportion killed of lymphoid cells from the *nu/nu* lymphatic rudiment and of BALB/c thymocytes was comparably high (80–90%), (Fig. 2). Absorption studies would be needed, however, before quantitative statements could be made about the average amount of θ antigen on the different cells examined.

In electron microscope and semi-thin sections the rudiment contained typical thymic lymphocytes (mostly of the dark variety) in nests and layers; among them mesenchymal reticulum cells, occasional macrophages, groups of epithelial cells with electron-dense granules, clear vesicles, lipid-containing vesicles and accumulated cysts were observed. The epithelial cystic cells resembled the thymic epithelial cystic cells. A few plasma cells and mast cells were also seen. The entire tissue was transversed with numerous blood capillaries and (in the deeper or central part) postcapillary venules with very high endothelium containing many lymphocytes in the passage from or into the lumen (Figs 3–5).

These observations indicate that *nu/nu* mice of different genetic background have a lymphatic rudiment containing θ-positive lymphocytes and secretory epithelial cells in the thymic region. It may be the organ responsible for the generation of T cells established in the *nu/nu* spleen. This organ

Fig. 5 Electron microscope appearance of the lymphoepithelial organ of a *nu/nu* (CBA) male aged 55 d. Lymphocytes surrounding a cystic epithelial cell (CEp). ×5,540.

does not have all the histological attributes of a well-developed mammalian thymus; in its organisation it is more like the thymus of lower vertebrates. Ontogenetically, it may be a true derivative of the branchial pouch III entoderm, whereas the polycystic organ may represent the aberrant development of thymus IV only[5], or of the ectodermal component (ductus ectobranchialis). The polycystic structures can be found also in *nu/+* hybrids with normal thymus development[2], and inside or outside the thymus of normal euthymic inbred mice (M.H., Vanecek, and P.R., unpublished).

M. HOLUB
P. ROSSMANN
H. TLASKALOVA
H. VIDMAROVA

*Institute for Clinical and Experimental
Medicine and Institute
for Microbiology CSAV,
Prague 4, Czechoslovakia*

1 Pantelouris, E. M., and Hair, J , *J. Embryol. exp Morph.*, 24, 615 (1970)
2 Groscurth, P , Munterer, M., and Tondury, G., *Proc. first Int. Workshop on Nude Mice* (edit. by Rygaard, J , and Povlsen, C. O.), 31 (Fischer, Stuttgart, 1974).
3 Cordier, A C., *J. ultrastruc. Res* , 47, 26 (1974)
4 Tamaoki, N., and Esaki, K., *Proc first Int. Workshop on Nude Mice* (edit. by Rygaard, J , and Povlsen, C. O.), 43 (Fischer, Stuttgart, 1974).
5 Kindred, B., and Loor, F., *J. exp. Med* , 139, 1215 (1974)
6 Loor, F., and Roelants, G. E., *Nature*, 251, 229 (1974).
7 Pritchard, H., and Micklem, H S , *Proc. first Int. Workshop on Nude Mice* (edit. Rygaard, J., and Povlsen, C. O), 127 (Fischer, Stuttgart, 1974)
8 Reif, A. E., and Allen, J. M., *Nature*, 209, 521 (1966)
9 Hair, J , *Proc first Int. Workshop on Nude Mice* (edit. Rygaard, J , and Povlsen, C. O), 23 (Fischer, Stuttgart, 1974).

Striated muscle fibres differentiate in monolayer cultures of adult thymus reticulum

THE thymus[1] is credited with a central role in the generation of clonal diversity of lymphocytes[2], and it may perform a critical function in the generation and(or) prevention of autoimmune disorders[3]. Less attention has been paid to other, non-immunological thymus functions, such as control of haemo-poiesis[4], development of certain leukaemias[5], and regulatory roles in the organism's hormonal balance[6].

We have described a tissue culture method that allows growth *in vitro* of thymus reticulum monolayers derived from

adult normal rats or mice[7]. These cultures at early stages consist of two main cell types, reticular and epithelial. In this communication we report the clonal differentiation of a third cell type, namely fully differentiated, striated muscle fibres in thymus reticulum cultures. This cell system, besides offering an approach to the analysis of muscle differentiation from adult mammalian precursors, has interesting biological and clinical implications.

The preparation of the cultures has been described in detail elsewhere[7]. Thymuses from young adult inbred Lewis rats (4–6 months) or C3Heb mice (4–6 weeks) were excised and carefully trimmed free of adjacent connective tissue as well as of the surface capsule. They were then minced with scissors, rinsed with phosphate-buffered saline (PBS), and subjected to differential trypsinisation to increase the concentration of the reticulum cells. The dissociated cells were suspended in Waymouth's medium enriched with 5% postnatal calf serum and plated into plastic Petri dishes (Fa. Greiner, Nürtingen), at a concentration of 30×10^6 cells ml^{-1}. In later experiments, we used gelatin-coated dishes[8]. After 2–3 d, most of the lymphoid cells were eliminated because of the relatively poor initial culture medium. The supernatant liquid containing the cellular debris was discarded and the cultures were replenished with Eagle's medium containing 15% horse serum. The medium was changed every 4 d.

During the first days of culture, single adhering thymus reticulum cells begin to divide and form confluent monolayers by day 7. In more than 95% of the cultures (about 150 independent platings) we found small spindle-shaped cells forming clones and fusing to contracting muscle fibres from day 8–10 (Fig. 1). These clones closely resemble those seen in myoblast cultures derived from chicken and rat embryos[8,9]. To demonstrate myosin biosynthesis, duplicate cultures of varying ages were labelled with 3.75 μCi ^{14}C-leucine (342 μCi $mmol^{-1}$) per dish for 2 h in leucine-free minimal essential medium. Myosin was extracted and assayed as described elsewhere[10]. Table 1 demonstrates that concomitantly with muscle fibre fusion, myosin synthesis is initiated.

Table 1 Myosin biosynthesis by thymus muscle clones

	Days *in vitro*	Myosin ^{14}C (c.p.m per 100-mm dish)
a	7	1,175 (background)
	7	1,476 (background)
b	12	25,457
	12	27,482

a, No fibre colonies present in the cultures; *b*, fibre colonies present in the cultures.

The origin of the thymus-derived striated muscles remains an open question. Trivially, these muscle cells could have developed from myoblasts in the connective tissue 'contaminating' the thymus reticulum cultures. To test this possibility, we removed not only the thymus capsules but also the adjacent cortical tissue and established parallel cultures from this material as well as from the rest of the thymus gland. The majority of the muscle clones arose from the latter starting material. Since the cortical fraction contains a higher proportion of connective tissue cells than the medullar fraction, it is probable that the muscle cells are derived from a genuine thymus component. This is corroborated by our failure to establish muscle cultures from non-thymic organs such as spleen, bone marrow, liver and kidney (H. W., unpublished).

Mammalian thymuses could contain committed myoblasts as physiological components. Indeed, myoid cells have been described in very early studies[11]. Striated muscle was observed in reptile and avian thymus[12,13]. The fact that there are but very few claims that myoid cells exist in normal adult mam-
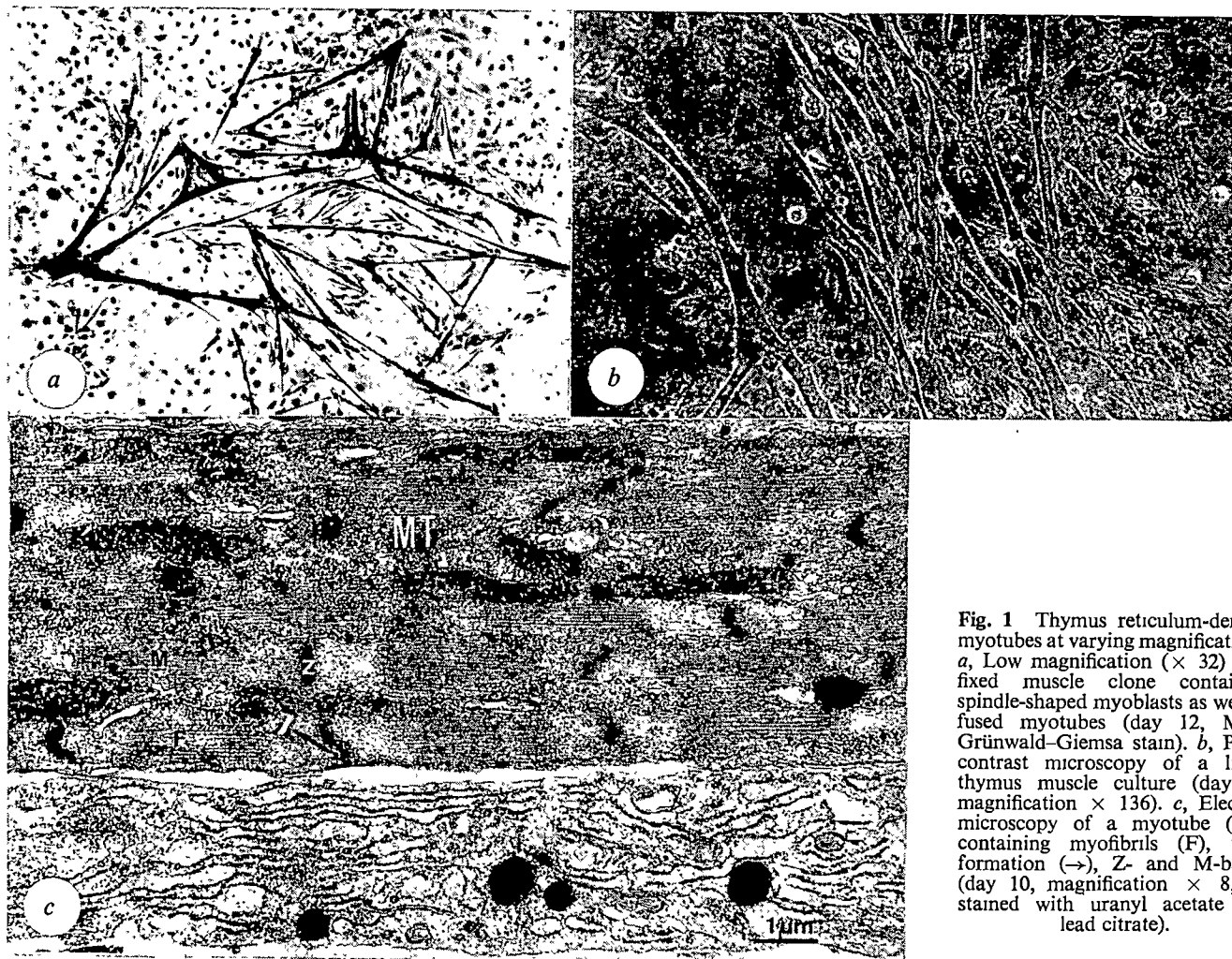
Fig. 1 Thymus reticulum-derived myotubes at varying magnifications. *a*, Low magnification ($\times$ 32) of a fixed muscle clone containing spindle-shaped myoblasts as well as fused myotubes (day 12, May–Grünwald–Giemsa stain). *b*, Phase contrast microscopy of a living thymus muscle culture (day 10, magnification $\times$ 136). *c*, Electron microscopy of a myotube (MT) containing myofibrils (F), triad formation ($\rightarrow$), Z- and M-bands (day 10, magnification $\times$ 8,925, stained with uranyl acetate and lead citrate).

malian organs[14] might argue against the possibility that thymus muscle clones are derived from pre-existing committed precursor cells.

Thus, we are left with the third possibility, namely, differentiation of thymus muscle clones from pluripotential stem cells. Several arguments seem to favour this possibility. It has been shown that, apart from muscle, tissues as different as osteocytes[15] and chondrocytes (D. Yaffe, personal communication) can differentiate from thymus cells in particular conditions *in vitro*. In our cultures, the relatively long latency period of 8 d preceding muscle cell fusion could be due to differentiation processes of originally pluripotential stem cells. This is well in line with the strikingly similar behaviour of mouse teratoma cells. Undifferentiated OTT 6050 embryoid body cells differentiate into muscle tubes when they are cultured in very similar conditions to our thymus cells[16].

The relationship of thymic cells and neoplastic pluripotential stem cells may be more than coincidental. It should be noted that the anterior mediastinal space shows the highest rate of teratomas, next to the gonads, and that these teratomas are thought to originate from thymic tissue[17]. It is tempting to speculate that mediastinal teratomas arise from neoplastic transformation of pluripotential stem cells in the thymus. Such stem cells, accumulated in this organ, may have a physiological role in the regeneration of the body's tissues. Experimental studies of congenital aplasias, for example in nude mice, should yield further information.

A final, very obvious implication could concern the correlation of thymic lesions and muscle autoimmune diseases like myasthenia gravis in particular. As we demonstrated before, thymocytes can be *in vitro* sensitised against autochthonous thymus reticulum cells[3]. It seems possible, therefore, that, following a defect of control, thymus lymphocytes could be

autosensitised against a thymus component bearing muscle antigens, and that such autosensitised lymphocytes cause the pathological lesions leading to myasthenia. Whether or not this is the case, our culture system provides the means to approach this problem experimentally.

H. WEKERLE
B. PATERSON*
U.-P. KETELSEN
M. FELDMAN

*Max-Planck Institut für Immunbiologie,
Freiburg-Zähringen, West Germany
and Department of Cell Biology,
The Weizmann Institute of Science,
Rehovot, Israel*

1 Raff, M , *Transplant. Rev.*, **6**, 52 (1971).
2 Jerne, N. K , *Eur. J. Immun.*, **1**, 1 (1975).
3 Cohen, I. R , and Wekerle, H., *Science*, **176**, 1324 (1972).
4 Resnitzky, P., Zipori, D., and Trainin, N , *Blood*, **37**, 634 (1971).
5 Kaplan, H S., *Cancer Res.*, **27**, 1325 (1967).
6 Pierpaoli, W., and Sorkin, E , *Nature*, **238**, 282 (1972)
7 Wekerle, H., Cohen, I R., and Feldman, M , *Eur J. Immun* , **3**, 645 (1973)
8 Hauschka, S. D , in *Growth, Nutrition and Metabolism of Cells in Cultures* (edit by Rothblat, G. H , and Cristofalo, V. J.), **2**, 84 (Academic, New York and London, 1972).
9 Yaffe, D , *Expl Cell Res* , **66**, 33 (1971).
10 Paterson, B., and Strohman, R. C., *Devl Biol.*, **29**, 113 (1972)
11 Hammar, J A , *Anat. Anz.*, **37**, 97 (1905)
12 Raviola, E , and Raviola, G , *Am J Anat* , **121**, 623 (1967)
13 Van de Velde, R. L , and Friedman, N B., *Am J Path* , **59**, 347 (1970).
14 Henry, K , *Lancet*, **ii**, 638 (1968).
15 Friedenstein, A J., and Lalykina, K. S., *Eur. J Immun* , **2**, 602 (1972)
16 Gearhart, J. D , and Mintz, B , *Proc natn Acad Sci U.S A.*, **71**, 1734 (1974)
17 Schlumberger, H G , *Archs Path.*, **41**, 398 (1946)

Continuous cultures of fused cells secreting antibody of predefined specificity

THE manufacture of predefined specific antibodies by means of permanent tissue culture cell lines is of general interest. There are at present a considerable number of permanent cultures of myeloma cells[1,2] and screening procedures have been used to reveal antibody activity in some of them. This, however, is not a satisfactory source of monoclonal antibodies of predefined specificity. We describe here the derivation of a number of tissue culture cell lines which secrete anti-sheep red blood cell (SRBC) antibodies. The cell lines are made by fusion of a mouse myeloma and mouse spleen cells from an immunised donor. To understand the expression and interactions of the Ig chains from the parental lines, fusion experiments between two known mouse myeloma lines were carried out.

Each immunoglobulin chain results from the integrated expression of one of several V and C genes coding respectively for its variable and constant sections. Each cell expresses only one of the two possible alleles (allelic exclusion; reviewed in ref. 3). When two antibody-producing cells are fused, the products of both parental lines are expressed[4,5], and although the light and heavy chains of both parental lines are randomly joined, no evidence of scrambling of V and C sections is observed[4]. These results, obtained in an heterologous system involving cells of rat and mouse origin, have now been confirmed by fusing two myeloma cells of the same mouse strain,

The protein secreted (MOPC 21) is an IgG1 (κ) which has been fully sequenced[7,8]. Equal numbers of cells from each parental line were fused using inactivated Sendai virus[9] and samples contining 2×10^5 cells were grown in selective medium in separate dishes. Four out of ten dishes showed growth in selective medium and these were taken as independent hybrid lines, probably derived from single fusion events. The karyotype of the hybrid cells after 5 months in culture was just under the sum of the two parental lines (Table 1). Figure 1 shows the isoelectric focusing[10] (IEF) pattern of the secreted products of different lines. The hybrid cells (samples c–h in Fig. 1) give a much more complex pattern than either parent (a and b) or a mixture of the parental lines (m). The important feature of the new pattern is the presence of extra bands (Fig. 1, arrows). These new bands, however, do not seem to be the result of differences in primary structure; this is indicated by the IEF pattern of the products after reduction to separate the heavy and light chains (Fig. 1B). The IEF pattern of chains of the hybrid clones (Fig. 1B, g) is equivalent to the sum of the IEF pattern (a and b) of chains of the parental clones with no evidence of extra products. We conclude that, as previously shown with interspecies hybrids[4,5], new Ig molecules are produced as a result of mixed association between heavy and light chains from the two parents. This process is intracellular as a mixed cell population does not give rise to such hybrid molecules (compare m and g, Fig. 1A). The individual cells must therefore be able to express both isotypes. This result shows that in hybrid cells the expression of one isotype and idiotype does not exclude the expression of another: both heavy chain
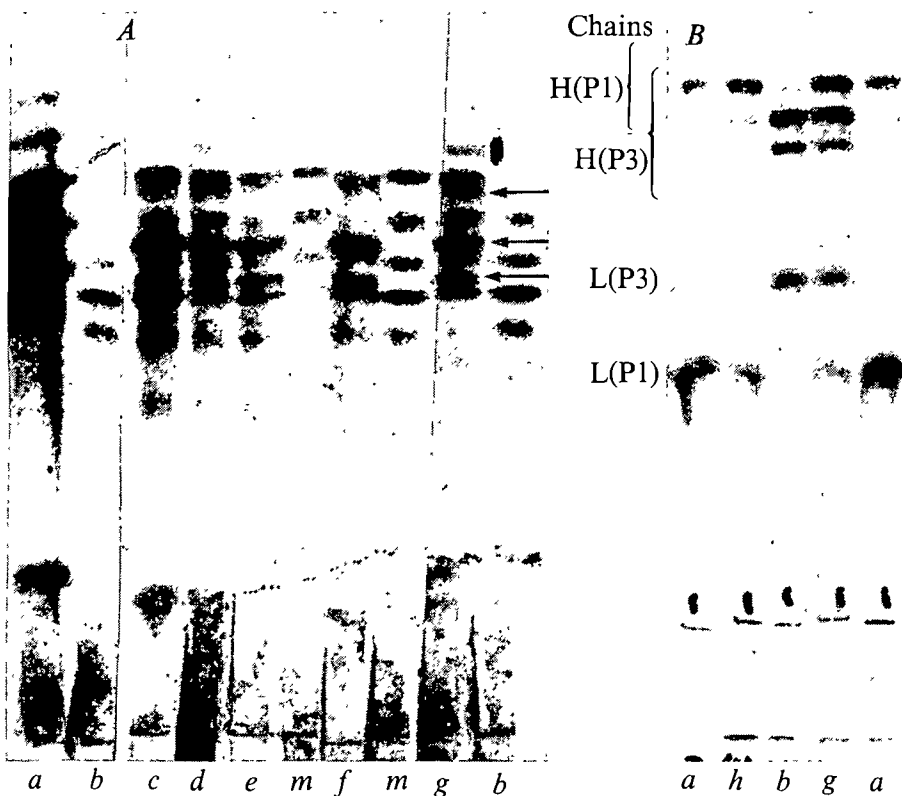
Fig. 1 Autoradiograph of labelled components secreted by the parental and hybrid cell lines analysed by IEF before (A) and after reduction (B). Cells were incubated in the presence of ^{14}C-lysine[14] and the supernatant applied on polyacrylamide slabs. A, pH range 6.0 (bottom) to 8.0 (top) in 4 M urea. B, pH range 5.0 (bottom) to 9.0 (top) in 6 M urea; the supernatant was incubated for 20 min at 37 °C in the presence of 8 M urea, 1.5 M mercaptoethanol and 0.1 M potassium phosphate pH 8.0 before being applied to the right slab. Supernatants from parental cell lines in: a, P1Bu1; b, P3-X67Ag8; and m, mixture of equal number of P1Bul and P3-X67Ag8 cells. Supernatants from two independently derived hybrid lines are shown: c–f, four subclones from Hy-3; g and h, two subclones from Hy-B. Fusion was carried out[4,9] using 10^6 cells of each parental line and 4,000 haemagglutination units inactivated Sendai virus (Searle). Cells were divided into ten equal samples and grown separately in selective medium (HAT medium, ref. 6). Medium was changed every 3 d. Successful hybrid lines were obtained in four of the cultures, and all gave similar IEF patterns. Hy-B and Hy-3 were further cloned in soft agar[14]. L, Light; H, heavy.

and provide the background for the derivation and understanding of antibody-secreting hybrid lines in which one of the parental cells is an antibody-producing spleen cell.

Two myeloma cell lines of BALB/c origin were used. P1Bul is resistant to 5-bromo-2'-deoxyuridine[4], does not grow in selective medium (HAT, ref. 6) and secretes a myeloma protein, Adj PC5, which is an IgG2A (κ), (ref. 1). Synthesis is not balanced and free light chains are also secreted. The second cell line, P3-X63Ag8, prepared from P3 cells[2], is resistant to 20 μg ml^{-1} 8-azaguanine and does not grow in HAT medium.

·sotypes (γ1 and γ2a) and both V_H and both V_L regions (idiotypes) are expressed. There are no allotypic markers for the C_κ region to provide direct proof for the expression of both parental C_κ regions. But this is indicated by the phenotypic link between the V and C regions.

Figure 1A shows that clones derived from different hybridisation experiments and from subclones of one line are indistinguishable. This has also been observed in other experiments (data not shown). Variants were, however, found in a survey of 100 subclones. The difference is often associated with changes
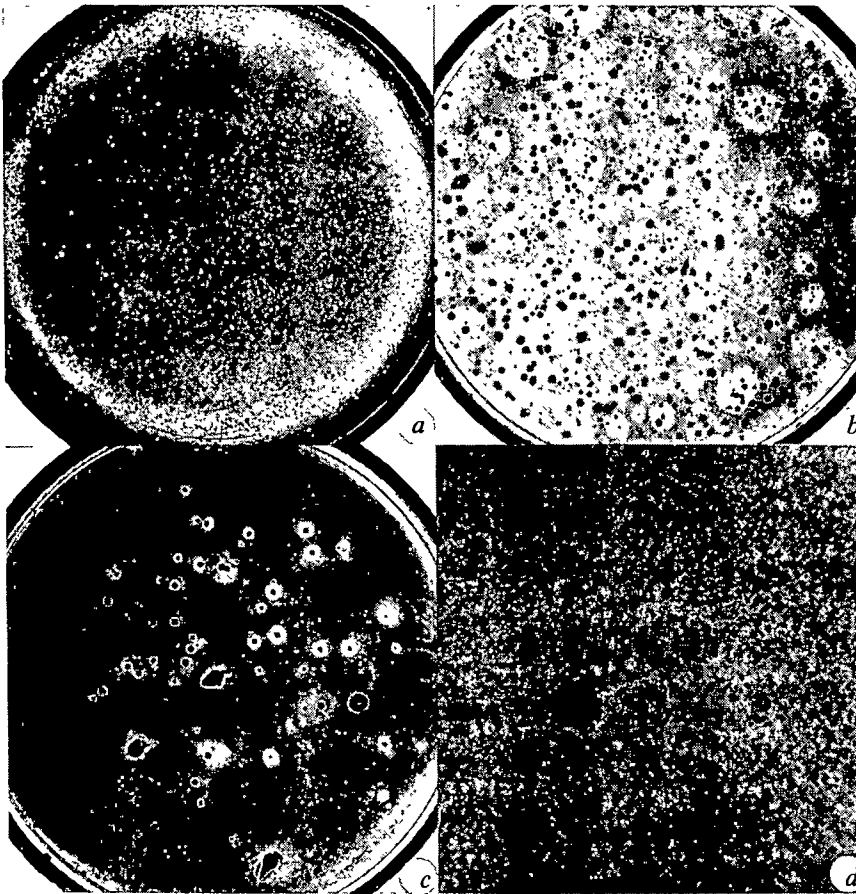
Fig. 2 Isolation of an anti-SRBC antibody-secreting cell clone. Activity was revealed by a halo of haemolysed SRBC. Direct plaques given by: *a*, 6,000 hybrid cells Sp-1; *b*, clones grown in soft agar from an inoculum of 2,000 Sp-1 cells; *c*, recloning of one of the positive clones Sp-1/7; *d*, higher magnification of a positive clone. Myeloma cells (10⁷ P3-X67A g8) were fused to 10⁸ spleen cells from an immunised BALB/c mouse. Mice were immunised by intraperitoneal injection of 0.2 ml packed SRBC diluted 1:10, boosted after 1 month and the spleens collected 4 d later. After fusion, cells (Sp-1) were grown for 8 d in HAT medium, changed at 1–3 d intervals. Cells were then grown in Dulbecco modified Eagle's medium, supplemented for 2 weeks with hypoxanthine and thymidine. Forty days after fusion the presence of anti-SRBC activity was revealed as shown in *a*. The ratio of plaque forming cells/total number of hybrid cells was 1/30. This hybrid cell population was cloned in soft agar (50% cloning efficiency). A modified plaque assay was used to reveal positive clones shown in *b–d* as follows. When cell clones had reached a suitable size, they were overlaid in sterile conditions with 2 ml 0 6% agarose in phosphate-buffered saline containing 25 µl packed SRBC and 0.2 ml fresh guinea pig serum (absorbed with SRBC) as source of complement. *b*, Taken after overnight incubation at 37 °C. The ratio of positive/total number of clones was 1/33. A suitable positive clone was picked out and grown in suspension. This clone was called Sp-1/7, and was recloned as shown in *c*; over 90% of the clones gave positive lysis. A second experiment in which 10⁶ P3-X67Ag8 cells were fused with 10⁸ spleen cells was the source of a clone giving rise to indirect plaques (clone Sp-2/3-3). Indirect plaques were produced by the addition of 1:20 sheep anti-MOPC 21 antibody to the agarose overlay.

in the ratios of the different chains and occasionally with the total disappearance of one or other of the chains. Such events are best visualised on IEF analysis of the separated chains (for example, Fig. 1*h*, in which the heavy chain of P3 is no longer observed). The important point that no new chains are detected by IEF complements a previous study[4] of a rat–mouse hybrid line in which scrambling of *V* and *C* regions from the light chains of rat and mouse was not observed. In this study, both light chains have identical C_κ regions and therefore scrambled V_L–C_L molecules would be undetected. On the other hand, the heavy chains are of different subclasses and we expect scrambled V_H–C_H to be detectable by IEF. They were not observed in the clones studied and if they occur must do so at a lower frequency. We conclude that in syngeneic cell hybrids (as well as in interspecies cell hybrids) *V*–*C* integration is not the result of cytoplasmic events. Integration as a result of DNA translocation or rearrangement during transcription is also suggested by the presence of integrated mRNA molecules[11] and by the existence of defective heavy chains in which a deletion of *V* and *C* sections seems to take place in already committed cells[12].

The cell line P3-X63Ag8 described above dies when exposed to HAT medium. Spleen cells from an immunised mouse also die in growth medium. When both cells are fused by Sendai virus and the resulting mixture is grown in HAT medium, surviving clones can be observed to grow and become established after a few weeks. We have used SRBC as immunogen, which enabled us, after culturing the fused lines, to determine the presence of specific antibody-producing cells by a plaque assay technique[13] (Fig. 2*a*). The hybrid cells were cloned in soft agar[14] and clones producing antibody were easily detected by an overlay of SRBC and complement (Fig. 2*b*). Individual clones were isolated and shown to retain their phenotype as almost all the clones of the derived purified line are capable of lysing SRBC (Fig. 2*c*). The clones were visible to the naked eye (for example, Fig. 2*d*). Both direct and indirect plaque

assays[13] have been used to detect specific clones and representative clones of both types have been characterised and studied.

The derived lines (Sp hybrids) are hybrid cell lines for the following reasons. They grow in selective medium. Their karyotype after 4 months in culture (Table 1) is a little smaller than the sum of the two parental lines but more than twice the chromosome number of normal BALB/c cells, indicating that the lines are not the result of fusion between spleen cells. In addition the lines contain a metacentric chromosome also present in the parental P3-X67Ag8. Finally, the secreted immunoglobulins contain MOPC 21 protein in addition to new, unknown components. The latter presumably represent the chains derived from the specific anti-SRBC antibody. Figure 3*A* shows the IEF pattern of the material secreted by two such Sp hybrid clones. The IEF bands derived from the parental P3 line are visible in the pattern of the hybrid cells, although obscured by the presence of a number of new bands. The pattern is very complex, but the complexity of hybrids of this type is likely to result from the random recombination of chains (see above, Fig. 1). Indeed, IEF patterns of the reduced material secreted by the spleen–P3 hybrid clones gave a simpler pattern of Ig chains. The heavy and light chains of the P3 parental line became prominent, and new bands were apparent.

The hybrid Sp-1 gave direct plaques and this suggested that it produces an IgM antibody. This is confirmed in Fig. 4 which shows the inhibition of SRBC lysis by a specific anti-IgM

Table 1 Number of chromosomes in parental and hybrid cell lines

Cell line	Number of chromosomes per cell	Mean
P3-X67Ag8	66,65,65,65,65	65
P1 Bu1	Ref. 4	55
Mouse spleen cells	—	40
Hy-B (P1–P3)	112,110,104,104,102	106
Sp-1/7-2	93,90,89,89,87	90
Sp-2/3-3	97,98,96,96,94,88	95

antibody. IEF techniques usually do not reveal 19S IgM molecules. IgM is therefore unlikely to be present in the unreduced sample *a* (Fig. 3*B*) but μ chains should contribute to the pattern obtained after reduction (sample *a*, Fig. 3*A*).

The above results show that cell fusion techniques are a powerful tool to produce specific antibody directed against a predetermined antigen. It further shows that it is possible to isolate hybrid lines producing different antibodies directed against the same antigen and carrying different effector functions (direct and indirect plaque).

The uncloned population of P3–spleen hybrid cells seems quite heterogeneous. Using suitable detection procedures it should be possible to isolate tissue culture cell lines making different classes of antibody. To facilitate our studies we have used a myeloma parental line which itself produced an Ig. Variants in which one of the parental chains is no longer expressed seem fairly common in the case of P1–P3 hybrids (Fig. 1*h*). Therefore selection of lines in which only the specific antibody chains are expressed seems reasonably simple. Alternatively, non-producing variants of myeloma lines could be used for fusion.

We used SRBC as antigen. Three different fusion experiments were successful in producing a large number of antibody-producing cells. Three weeks after the initial fusion, 33/1,086

A   B

Fig. 3 Autoradiograph of labelled components secreted by anti-SRBC specific hybrid lines Fractionation before (*B*) and after (*A*) reduction was by IEF *p*H gradient was 5 0 (bottom) to 9 0 (top) in the presence of 6 M urea. Other conditions as in Fig. 1. Supernatants from: *a*, hybrid clone Sp-1/7-2; *b*, hybrid clone Sp-2/3-3; *c*, myeloma line P3-X67Ag8.

Fig. 4 Inhibition of haemolysis by antibody secreted by hybrid clone Sp-1/7-2. The reaction was in a 9-cm Petri dish with a layer of 5 ml 0.6% agarose in phosphate-buffered saline containing 1/80 (v/v) SRBC Centre well contains 2.5 μl 20 times concentrated culture medium of clone Sp-1/7-2 and 2.5 μl mouse serum *a*, Sheep specific anti-mouse macroglobulin (MOPC 104E, Dr Feinstein); *b*, sheep anti-MOPC 21 (P3) IgG1 absorbed with Adj PC-5; *c*, sheep anti-Adj PC-5 (IgG2a) absorbed with MOPC 21. After overnight incubation at room temperature the plate was developed with guinea pig serum diluted 1:10 in Dulbecco's medium without serum.

clones (3%) were positive by the direct plaque assay. The cloning efficiency in the experiment was 50%. In another experiment, however, the proportion of positive clones was considerably lower (about 0.2%). In a third experiment the hybrid population was studied by limiting dilution analysis. From 157 independent hybrids, as many as 15 had anti-SRBC activity. The proportion of positive over negative clones is remarkably high. It is possible that spleen cells which have been triggered during immunisation are particularly successful in giving rise to viable hybrids. It remains to be seen whether similar results can be obtained using other antigenes.

The cells used in this study are all of BALB/c origin and the hybrid clones can be injected into BALB/c mice to produce solid tumours and serum having anti-SRBC activity. It is possible to hybridise antibody-producing cells from different origins[4,5]. Such cells can be grown *in vitro* in massive cultures to provide specific antibody. Such cultures could be valuable for medical and industrial use.

G. Köhler
C. Milstein

MRC Laboratory of Molecular Biology,
Hills Road, Cambridge CB2 2QH, UK

[1] Potter, M , *Physiol Rev* , 52, 631-719 (1972).
[2] Horibata, K., and Harris, A W., *Expl Cell Res* , 60, 61-70 (1970).
[3] Milstein, C , and Munro, A. J., in *Defence and Recognition* (edit. by Porter, R R.), 199-228 (MTP Int Rev. Sci , Butterworth, London, 1973).
[4] Cotton, R G H , and Milstein. C , *Nature*, 244, 42-43 (1973)
[5] Schwaber, J , and Cohen, E. P., *Proc. natn. Acad. Sci U.S.A.*, 71, 2203-2207 (1974)
[6] Littlefield, J. W , *Science*, 145, 709 (1964).
[7] Svasti, J , and Milstein, C , *Biochem J* , 128, 427-444 (1972)
[8] Milstein, C., Adetugbo, K , Cowan, N. J , and Secher, D S , *Progress in Immunology*, II, 1 (edit by Brent, L., and Holborow, J), 157-168 (North-Holland, Amsterdam, 1974)
[9] Harris, H , and Watkins J F , *Nature*, 205, 640-646 (1965).
[10] Awdeh, A. L , Williamson, A R , and Askonas, B A., *Nature*, 219, 66-67 (1968)
[11] Milstein, C., Brownlee, G. G , Cartwright, E. M., Jarvis, J. M., and Proudfoot, N J , *Nature*, 252, 354-359 (1974).
[12] Frangione, B , and Milstein, C., *Nature*, 244, 597-599 (1969).
[13] Jerne, N K , and Nordin, A A , *Science*, 140, 405 (1963)
[14] Cotton, R. G H , Secher, D S , and Milstein, C , *Eur J. Immun* , 3, 135-140 (1973).

Naturally occurring cytotoxic tumour reactive antibodies directed against type C viral envelope antigens

TYPE C RNA viruses are frequently present in murine tumours, but the nature of the association between these viruses and malignancy is uncertain. The oncogene theory[1,2] contends that type C viruses possess oncogenic information and that malignancy is the result of activation of this genetic information. Alternatively, the expression of type C viruses in a tumour may be a consequence, rather than a cause, of the neoplastic change and may be beneficial to the host by providing for potential immunological detection and eradication of neoplastic cells[3]. If type C viral antigens are targets for an immune surveillance mechanism, then normal animals would be expected to show evidence of immune sensitisation against viral antigens expressed on the surface of tumour cells. Here, we present evidence that naturally occurring antibodies (NOA) cytotoxic for a type C virus releasing teratoma-derived cell line are directed against viral antigens.

TerA is one of several cell lines derived from a spontaneous ovarian teratoma of a C3H/HeIcrf mouse. NB1 is a cell line derived from a neuroblastoma adrenal metastasis which was present in the mouse bearing the primary ovarian teratoma. Wheras electron microscopic examination of TerA cells revealed abundant budding type C virus particles, no budding type C viruses were observed in electron micrographs of NB1 cells. Supernatant fluid from
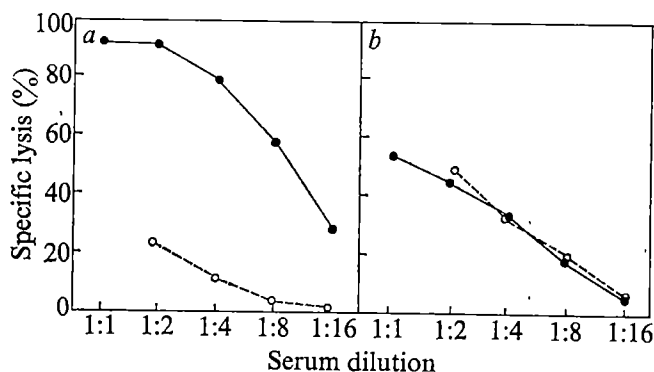
Fig. 1 Cytotoxic activity of normal serum of C3Hf/He mice unabsorbed (●) or absorbed with purified virus from TerA cells (○) and tested on either TerA (a) or NB1 cells (b). Virus was pelleted by ultracentrifugation and banded in a sucrose gradient using routine procedures. Briefly, supernatant fluid was collected 24 h after the cells were fed, was clarified by Millipore filtration (0.45 μm Millipore filter) and, after the addition of glycerin (6% final concentration), ultracentrifuged. Virus pellet was banded in a 20–60% linear sucrose gradient. It banded in a fraction with a density of 1.153 g ml⁻¹. This fraction was diluted with BSS and the virus reconcentrated by Amicon filtration (XM100 filter). Negative-staining electron microscopy revealed intact viruses with no discernible cellular debris. A 100 μl of volume of BSS containing an estimated 10¹⁰ sucrose-gradient-banded viruses from TerA cells was added to an equal volume of undiluted mouse serum. The mixture was incubated at 37 °C for 60 min and at 25 °C for 60 min. The tubes were centrifuged at 8,000g for 5 min and the supernatant serum tested for residual cytotoxic activity. Control sera were incubated with BSS. Cytotoxic activity was determined by a two-step RC′-dependent cytotoxicity assay as described previously[6–8]. Normal mouse serum in the absence of RC′ and RC′ in the absence of normal mouse serum were not cytotoxic for either TerA or NB1 cells. The specific antibody-mediated lysis (%) was calculated by the formula:

$$\frac{(\% \text{ Cytotoxicity mouse serum}+\text{RC}')-(\text{RC}'\text{ control})}{\text{Maximum release}-\text{RC}'\text{ control}} \times 100$$

In all experiments TerA-derived virus specifically removed more than 80% of the cytotoxic activity of normal serum for TerA cells.
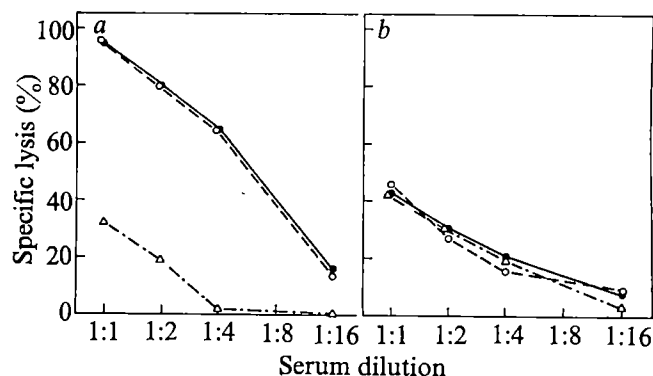
Fig. 2 Cytotoxic activity of normal mouse serum of C3Hf/He mice unabsorbed (●) or absorbed with either uninfected (○) or TerA virus-infected (△) SC1 cells, tested against TerA (a) or NB1 cells (b). Supernatants from TerA cell cultures were added to subconfluent cultures of SC1 cells. Cultures were treated as described in Table 1 and supernatants were assayed for reverse transcriptase as described previously[4]. Supernatant fluid from the infected SC1 cell lines was positive for reverse transcriptase activity (15 pmol ml⁻¹), whereas supernatant fluid from uninfected SC1 cell lines contained no reverse transcriptase activity. Undiluted normal mouse serum was absorbed for 40 min at 4 °C with a one-third volume of SC1 cells, either uninfected or infected 9 d previously with TerA-derived virus, and was tested for residual cytotoxicity against TerA and NB1 cells. Similar results have been obtained in two other experiments

TerA and NB1 cell cultures was assayed for RNA-dependent DNA polymerase (reverse transcriptase) activity and DNA-dependent DNA polymerase activity using the conditions described by Ross *et al.*[4]. The pelletable RNA-dependent DNA polymerase activity (pmol ³H-thymidine monophosphate incorporated per ml supernatant fluid) in supernatant fluid from TerA cell cultures was 20.83. The reverse transcriptase activity in supernatant fluid from NB1 cell cultures was −0.02. Supernatants from these cell lines did not contain significant levels of DNA-dependent DNA polymerase activity. Host range studies on TerA-derived virus indicated that the virus grew readily on the mouse indicator cell line SC1 but not on the rabbit cell line SIRC. In addition, the virus showed a 100-fold preference for replication in embryo-derived cultures from NIH Swiss mice compared with similar cultures derived from BALB/c mice in the XC cell assay[5]. The virus can, therefore, be classified as an N-tropic endogenous virus[5].

Normal sera of C3Hf/He mice were tested in neutralisation and in radioimmune precipitation assays for NOA reactive with the TerA-derived type C virus. A 1 : 10

Table 1 Neutralisation of infectivity of TerA-derived virus for SC1 by normal mouse serum

Dilution of supernatant containing TerA virus	Dilution of normal mouse serum	RNA-dependent DNA polymerase activity (pmol)
1 : 1	Nil	25.58 ± 0.42
1 : 10	Nil	21.24 ± 0.12
1 : 100	Nil	7.74 ± 0.91
1 : 1	1 : 10	1.44 ± 0.09
1 : 1	1 : 100	16 24 ± 1.08

Supernatant fluid from TerA cultures was filtered through a 0.45 μm Millipore filter. To aliquots of filtered medium was added normal mouse serum to final concentrations of 1 : 10 and 1 : 100. The medium was incubated at 37 °C for 1 h and refiltered through a 0.45 μm Millipore filter. Aliquots (2 ml) of untreated and mouse serum-treated undiluted TerA virus containing supernatants were added to subconfluent cultures of SC1 cells (2 × 10⁵ cells in 30 ml Falcon T flasks). In addition 2-ml aliquots of 1 : 10 and 1 : 100 dilution of TerA supernatant was added to SC1 cells, and the medium changed on day 3. Cells were subcultured on day 5 and the medium changed on day 8. Supernatant fluid was collected on day 9 and assayed for reverse transcriptase activity (pmol ³H-thymidine monophosphate incorporated per ml supernatant fluid) as described previously[4].

dilution of normal mouse serum markedly reduced the infectivity of the virus for SC1 cells. Significant neutralisation was also observed at a 1 : 100 dilution of mouse serum (Table 1). A radioimmune precipitation assay, using goat anti-mouse immunoglobulin antisera to precipitate ^{3}H-uridine-labelled virus bound to mouse immunoglobulin, confirmed that normal mouse sera contained NOA reactive with the type C virus (Table 2).

TerA and NB1 cells can be readily lysed in the presence of rabbit complement (RC') by NOA present in sera of C3Hf/He mice[6,7]. The antigens recognised by NOA on TerA and NB1 cells are, however, distinct. To determine whether NOA reactive with TerA cells were reactive with the type C virus produced by TerA cells, normal serum of C3Hf/He mice was preincubated with sucrose gradient banded TerA-derived virus before testing for residual cytotoxicity against TerA and NB1 cells. As shown in Fig. 1, absorption with purified virus greatly reduced the cytotoxicity of normal mouse serum against TerA cells but

---

Table 2 Radioimmune precipitation of ^{3}H-uridine labelled TerA-derived virus by normal mouse serum

Dilution of mouse serum	Radioactivity (%) bound by serum immunoglobulin
1 : 1	83.3 ± 3 4
1 : 5	62.6 ± 4.4
1 : 25	42.0 ± 5.0
1 : 125	2.1 ± 1.7

TerA-derived virus was labelled with ^{3}H-uridine (New England Nuclear) by incubating TerA cells with 10 μCi ml^{-1} isotope for 24 h. Supernatant fluid (10 ml) was collected and filtered through a 0.45 μm Millipore filter The virus was pelleted by ultracentrifugation and resuspended to a 100 μl volume, containing approximately 10^{10} viral particles A 10 μl volume of either undiluted or diluted mouse serum or of balanced salt solution (BSS) was added to 10 μl virus suspension and the mixture was incubated at 37 °C for 1 h and at 25 °C for 1 h. A volume (30 μl) of goat anti-mouse immunoglobulin antiserum (Dr R. Asofsky. NIH) was added to the tubes. A 10 μl volume of BSS was added to the tubes containing undiluted mouse serum and virus and 10 μl normal mouse serum was added to tubes originally containing either BSS or diluted mouse serum. A precipitate developed in each tube. After 2 h incubation at 25 °C the tubes were centrifuged at 1,000g for 10 min. A 20 μl aliquot from each tube was mixed with cold 10% TCA. Precipitates were collected on Millipore filters and assayed for radioactivity. Duplicate determinations were carried out on each serum dilution. Results are mean ± s. e. of values obtained in three separate experiments, and are expressed as a percentage reduction in supernatant radioactivity in tubes initially containing virus plus mouse serum compared with tubes initially containing virus plus BSS.

---

caused no significant reduction of anti-NB1 cytotoxicity. In some experiments, absorption of normal mouse serum with purified TerA-derived type C virus completely removed cytotoxicity against TerA cells. In addition, when normal mouse sera were absorbed with either uninfected SC1 cells or SC1 cells infected with TerA-derived virus and tested for residual cytotoxicity against TerA and NB1 cells, virus-infected SC1 cells specifically absorbed anti-TerA cytotoxicity (Fig. 2).

Our data indicate that NOA capable of binding intact type C virus released from TerA cells, and therefore presumably directed against viral envelope antigens (VEA), is cytotoxic, in the presence of RC', for TerA cells This finding contrasts with a report in which cytotoxicity was not demonstrated with NOA, shown by radioimmune precipitation assay to bind VEA of the Gross virus released from a cell line of AKR mice[9]. These authors used a single step cytotoxicity assay, rather than the more sensitive two-step assay used here In an immunoelectron microscopic study NOA were shown to react with both the cell surface of myeloma cells and with VEA of the type C virus released from the cells[10]. It could not be concluded, however, whether the cell surface antigen and the VEA recognised

by NOA were identical or whether the normal serum contained two populations of NOA recognising distinct antigens on the cell surface and on the viral envelope Sera of a variety of mouse strains are known to possess NOA capable of neutralising the xenotropic virus released from chemically treated cells of BALB/c mice[11]. These sera were not tested for reactivity with cell-surface antigens. Normal mouse. sera contain NOA reactive with a wide variety of tumour cell lines[6-8,12,13]. As partial expression of type C viral genetic information may occur, it cannot be excluded that NOA reactive with tumour cell lines which do not express type C viruses may not also be directed against a virally coded cell surface antigen. Although all tumour reactive NOA may not be directed against type C viral antigens, the findings reported in this paper are at least consistent with the notion that the selective expression of type C viral antigens by a tumour may assist in the immunological recognition of the tumour.

Sue Ellen Martin

Laboratory of Biochemical Genetics,
National Lung and Heart Institute

W. John Martin

National Cancer Institute,
National Institutes of Health,
Bethesda, Maryland 20014

1 Huebner, R. J., and Todaro, G J., *Proc. natn. Acad. Sci. U S A.*, 64, 1087–1090 (1969).
2 Todaro, G J., and Huebner, R. J., *Proc. natn. Acad. Sci. U.S.A.*, 69, 1009–1010 (1972).
3 Martin, W. J , *Cell Immun*, 15, 1–10 (1975).
4 Ross, J. E , Scolnick, E. M., Todaro, G. J., and Aronson, S.A., *Nature new Biol.*, 231, 163–167 (1971).
5 Rowe, W. P., Pugh, W E., and Hartley, J. W., *Virology*, 42, 1136–1139 (1970).
6 Martin, S. E., and Martin, W. J., *Proc. natn. Acad. Sci U.S.A.*, 72, 1036–1040 (1975).
7 Martin, S E., and Martin, W J., *J. Immun.* (in the press).
8 Martin, S. E., and Martin, W J , *Int J. Cancer*, 15, 658–664, (1975).
9 Hanna, M G., Ihle, J. N., Batzing, B. L , Tennant, R. W., and Schenley, C. K., *Cancer Res.*, 35, 164–171 (1975).
10 Herberman, R B , and Aoki, T., *J. exp Med.*, 136, 94–111 (1972)
11 Aaronson, S A., and Stephenson, J. R., *Proc natn Acad. Sci U S.A.*, 71, 1957–1961 (1974).
12 Sato, H , Boyse, E. A., Aoki, T , Iritani, C., and Old, L. J., *J. exp. Med*, 138, 593–606 (1973).
13 Martin, W. J., and Martin, S E , *Nature*, 249, 564–565 (1974).

Antigen formation in metal contact sensitivity

THERE have been reports of experimental induction of contact sensitivity to dinitrochlorobenzene (DNCB) and dinitrofluorobenzene by injection of dinitrophenol (DNP) conjugated to lymphoid cells[1-3]. The DNP group is known to be capable of acting as a hapten but it is difficult to apply the term 'hapten' to the case of inorganic metals which act as contact sensitisers since antibodies with specificity for the metal ions have not been demonstrated. This is hardly surprising in view of the small size of some of the metals implicated as sensitisers, yet metal salts have been shown to produce stimulation of lymphocytes from individuals with metal contact sensitivity[4-6]. The following experiments were designed to determine whether lymph node cells pulsed with beryllium were capable of inducing beryllium contact sensitivity.

Guinea pig lymph node cells or peripheral blood erythrocytes were incubated with 1 ml 500 μM beryllium sulphosalicylate (BeSSA, trace labelled with ^{7}Be) in saline for 1 h at 37 °C (ref. 6). Lymph node cell and erythrocyte concentrations were 2×10^7 and 10×10^7 cells ml^{-1}, respectively. Radioactivity was counted before and after washing three times with culture medium without added serum. This number of washes was chosen arbitrarily as radioactivity continued to be released from

the cells even after eight washes. The amount of BeSSA remaining with the cell suspension after three washes was found to vary with individual experiments from 3 to 30% of the original BeSSA added.

Beryllium-treated lymph node cells (4.0×10^7) in 4 ml medium were injected intraperitoneally into normal recipient guinea pigs. Erythrocytes (16.6 or 13.3×10^7) in 4 ml medium were used for injection.

Recipient guinea pigs were tested for contact sensitivity to beryllium 14 d later by painting the shaved back with 100 μl 1% beryllium fluoride (BeF$_2$) in methyl cellosolve–water–Tween 80 (45:45:10). Reactions were recorded at 24 and 48 h on an arbitrary scale 0–3 (ref. 7). Animals were classified as contact sensitive when skin test readings were at least 0.5 at both 24 and 48 h and when at least one of these readings had a value of 1 or more.

It was then necessary to determine what proportion of guinea pigs which had been used as recipients were capable of being sensitised to BeF$_2$ since only a proportion of outbred animals are potential responders[8]. Recipient guinea pigs were therefore painted on the left ear on 3 successive days with 15% BeF$_2$ in Triton X-100[6] and skin tested 14 d later with 1% BeF$_2$.

Table 1 Induction of contact sensitivity to BeF$_2$ by intraperitoneal injection of lymphocytes pulsed with BeSSA

Dose of BeSSA injected	Carrier	Skin test reactivity to 1% BeF$_2$	Sensitisation by painting with 15% BeF$_2$
0.22–0.33 μmol	4×10^7 lymphocytes (60–80% viable)	9/11	ND
0.32 μmol	13.3×10^7 16.7×10^7 erythrocytes	0/8	6/8
0.30 μmol	—	0/6	2/4 (2d)
0.03 μmol	—	0/4	4/4
—	4×10^7 lymphocytes	0/3	3/3

Outbred Dunkin–Hartley guinea pigs were used as both donors and recipients. ND, Not determined.

Table 1 shows that allogeneic lymph node cells pulsed with BeSSA were capable of inducing contact sensitivity to BeF$_2$ in normal recipient guinea pigs. Animals injected with an erythrocyte suspension containing a similar amount of beryllium did not develop contact sensitivity although 6 out of 8 recipients were shown subsequently to be capable of being sensitised. Similarly, guinea pigs injected with 0.03 or 0.3 μmol BeSSA in culture medium alone gave no reaction, suggesting that free BeSSA leaking from lymph node cells is unlikely to account for their efficacy in inducing sensitisation.

An attempt was made to induce beryllium contact sensitivity by injection of lymph node cells treated with BeSSA and killed by incubation with 1% sodium azide for 45 min at 37 °C. Cell viability after washing was less than 10%. Five allogeneic Dunkin–Hartley guinea pigs received 4×10^7 lymph node cells intraperitoneally but none became sensitised to BeF$_2$.

Table 2 Induction of contact sensitivity to BeF$_2$ in Dunkin–Harley guinea pigs by intraperitoneal injection of strain XIII lymphocytes pulsed with BeSSA

Recipients	Lymphocyte donors	Dose of BeSSA injected	Skin test reactivity to 1% BeF$_2$	Sensitisation by painting with 15% BeF$_2$
Dunkin–Hartley	Strain XIII	0.03–0.25 μmol	4/11	7/11
Strain XIII	Dunkin–Hartley	0.04–0 31 μmol	0/12	ND

ND, Not determined.

Table 2 shows results of experiments in which strain XIII guinea pigs, genetically unresponsive to beryllium by painting skin with BeF$_2$ (ref. 8), were used as donors or recipients for beryllium-treated cells. Strain XIII lymph node cells, pulsed with BeSSA, were found to be capable of inducing beryllium contact sensitivity in Dunkin–Hartley recipients. Strain XIII guinea pigs which had received Dunkin–Hartley lymph node cells showed no evidence of skin reactivity to BeF$_2$.

It is of considerable interest that lymph node cells treated with the chelated beryllium complex, BeSSA, induced beryllium contact sensitivity since beryllium in a highly chelated form has been shown to be ineffective in eliciting a skin reaction[9]. The fact that allogeneic cells were capable of inducing sensitisation would argue that in this system the injected cells were contributing to the formation of immunogenic conjugates rather than becoming actively allergised *in vitro* as suggested by Polak and Macher[3] using DNCB. The finding that strain XIII lymph node cells were also effective in inducing sensitivity would further support the contention that immunogen was being transferred. Negative results following injection of killed cells could be explained by an alteration of the antigen following azide treatment.

We interpret these experiments as demonstrating that antigen capable of inducing contact sensitivity to beryllium is formed when lymph node cells are pulsed *in vitro* with BeSSA. At present there is no information on whether this antigen is attached to the cells or is released from them as a complex. It is unlikely that BeSSA combining *in vivo* with tissues of the recipient will account for these results, since free BeSSA injected intraperitoneally failed to sensitise

JANET M. JONES
H. E. AMOS

*Department of Immunology,
Institute of Dermatology,
Homerton Grove,
London E9 6BX, UK*

1 Baumgarten, A., and Geczy, A F., *Immunology*, 19, 205–217 (1970).
2 McFarlin, D E, and Balfour, B , *Immunology*, 25, 995–1009 (1973)
3 Polak, L , and Macher, E., *Nature*, 252, 748–749 (1974).
4 MacLeod, T. M , Hutchinson, F , and Raffle, E J , *Br J. Derm* , 82, 487–492 (1970).
5 Polak, L and Frey, J R., *Int. Aichs Allergy*, 44, 161–171 (1974)
6 Jones, J. M , and Amos, H. E., *Int Archs Allergy*, 46, 161–171 (1974)
7 Frey, J. R , and Wenk, P., *Int Archs Allergy*, 11, 81–100 (1957)
8 Polak, L , Barnes, J M , and Turk, J. L , *Immunology*, 14, 707–711 (1968)
9 Krivanek, N , and Reeves, A. L., *Am ind Hyg Ass J.*, 33, 45–52 (1972)

Induced thermal resistance in HeLa cells

IN mammalian cells, hyperthermia causes temperature-dependent changes in cell growth parameters[1], reduces DNA and protein synthesis rates[2] and cell metabolism[3], and leads to a loss of proliferative capacity[4,5]. Harris[6] has shown that temperature-resistant pig kidney cells can be obtained after multiple exposures to very high thermal doses (colony-forming ability reduced to 10^{-5}–10^{-6} of controls). The purpose of our experiments was to determine whether a single hyperthermic treatment (44 °C for 1 h) could induce a state of thermotolerance in cells, and if so, what was the mode of origin of the thermal resistant cells.

HeLa cells (Flow Labs; Rockville, Maryland) were maintained in exponential growth at 37 °C in medium 199 supplemented with 10% foetal calf serum (both from Gibco). Their doubling time in these conditions was 23–24 h. Proliferative capacity, as measured by colony-forming ability *in vitro*, was used as a measure of survival. Thermal doses were administered to these exponentially growing cells by immersion of culture flasks in a temperature controlled water bath (temperature constant to ±0.1 °C) As it was known that nutritional factors are important in determining cellular response to hyperthermia[7], a standard treatment protocol was used with cells being treated in full medium 20 h after inoculation in the treatment flasks

After thermal treatments, cells were removed from the flasks by trypsinisation, combined with any floating cells, diluted and plated for colony formation in 5 ml fresh medium.

Figure 1 shows the survival response of exponentially growing HeLa cells treated at 44 °C for increasing times up to 3.5 h. Survival decreases exponentially as a function of time at 44 °C. This result is similar to that already reported[4,5,7], except that no (or a very small) shoulder region is apparent on the single dose–survival curve. Figure 1 also shows that the survival response is exponential even when survival is reduced to less than 0.1%. Whereas previous authors[4,5] have shown cell cycle-dependent fluctuations in thermal inactivation, this observation suggests that subpopulations have similar inactivation rates, as a resistant tail is not apparent as cell killing increases. Note that Westra and Dewey[4] have shown that the difference in thermal response for CHO cells comparing sensitive mitotic and S-phase cells and resistant G_1 cells was primarily the result of a decrease in the shoulder of the survival curves, whereas the slopes of the exponential portion of the curves did not change. Thus, whereas the heterogeneous cultures reported here could contain subpopulations, some or all of which might have shoulders, a composite survival curve would also have a shoulder. This lack of a shoulder on the single dose–survival curve shown in Fig. 1 implies that these HeLa cells do not accumulate sublethal hyperthermic damage.

When two-dose experiments were used to test for the recovery of our HeLa cells from sublethal hyperthermic damage (Fig. 2a), cell survival increased with increasing incubation at 37 °C between two thermal treatments of 44 °C for 1 h. This result is similar to that previously reported for HeLa S-3 cells by Palzer and Heidelberger[5] who interpreted their data to be recovery

Fig. 1 Response of HeLa cells to two-dose fractionated hyperthermic treatments (each dose 44 °C for 1 h). *a*, Cellular survival when incubated at 37 °C for various times between the two (44 °C for 1 h) thermal doses. *b*, Response of cells to fractionated thermal treatments with incubation first at 0 °C and then 37 °C between doses. In these experiments, cells were treated at 44 °C for 1 h. Immediately after this treatment, the cells were transferred to 0 °C. After 1 and 2 h at 0 °C, some treatment flasks were immediately returned to 44 °C for 1. After 2 h at 0 °C, the other treatment flasks were transferred to 37 °C and after various times at 37 °C, were reexposed to 44 °C for 1 h. After the second 44 °C treatment, cells were collected and plated for colony formation. Control experiments showed that the immediate transition of cells from 44 °C to 0 °C did not have a lethal effect on cells. Standard errors of survival determinations are as shown.

Fig. 1 Thermal survival response of exponentially growing HeLa cells. ●, Survival response of cells treated with single exposures of 44 °C for increasing time; ○, response of cells treated at 44 °C for 1 h, returned to the 37 °C incubator for 2 h and given second graded doses of 44 °C for increasing times. Standard errors of survival determinations are as shown

from sublethal damage. That the increase in survival in HeLa cells (Fig. 2a) is not recovery from sublethal damage, however, is supported by two factors. First, the absence of a shoulder on the single dose–survival curve implies no capacity for sublethal damage accumulation. Second, the results shown in Fig. 1 demonstrate that when HeLa cells are challenged with 44 °C for 1 h, incubated for 2 h at 37 °C and then given graded second thermal doses, again no shoulder is evident during 4 additional hours of treatment, whereas the sensitivity of the survival response, as measured by the slope, is appreciably changed.

A comparison of the slopes of thermal survival curves with D_0 being a measure of the time for survival to be reduced to $1/e$ on the exponential portion of the curve at a given temperature supports this observation. Thermal treatment at 44 °C for 1 h followed by 2 h incubation at 37 °C reduces the thermal sensitivity at 44 °C of our HeLa cells by a factor of three, with the D_0 for previously unheated cells being 0.5 h and the D_0 for previously heated cells being 1.5 h. Thus, these data show that the increase in survival seen in the two-dose experiments of Fig. 2a is actually the result of a decrease in the sensitivity of cells to the second dose.

The possibility existed that the reduced sensitivity (Fig. 1) could be the result of cell progression from a sensitive cell cycle phase to a less sensitive phase, as S-phase cells are more sensitive to thermal inactivation than G_1 cells[4,5]. Data to be published elsewhere, however, demonstrate that the thermal doses used in these survival studies inhibit cell progression of both G_1 and S-phase cells for longer periods than those described in Figs 1 and 2 This implies only minimal reassortment of cells

into different cell cycle phases during these experiments and argues against a major accumulation of cells into a resistant cell cycle phase.

The reduced thermal sensitivity of these HeLa cells, after an initial thermal dose, has two characteristics. First, the process(es) leading to the changed sensitivity does not become active at 44 °C, as the single dose–survival curve of Fig. 1 does not show a resistant tail when cells are maintained at 44 °C for 3.5 h. Second, the changed sensitivity becomes apparent only when the cells are returned to 37 °C for a time before subsequent heating. To test this latter point, additional two-dose experiments were carried out. All cells were treated at 44 °C for 1 h, after which cultures were immediately transferred to an ice bath at 0 °C for up to 2 h and then transferred to a 37 °C incubator, with the second 44 °C (1 h) thermal dose being delivered at the end of hourly intervals for up to 5 h total incubation between doses. Incubation at 0 °C (Fig. 2b), which inhibits cellular metabolic activity, inhibits the increase in survival (Fig. 2a) when cells are immediately returned to 37 °C between the two hyperthermic treatments. When cells are returned to 37 °C after 2 h at 0 °C between doses, survival increases rapidly and reaches the same survival level 2 h later as cells incubated at 37 °C immediately after the initial thermal dose.

These results suggest that the thermal resistance seen in Fig. 1 is induced by the first thermal dose, is dependent on cell metabolism and in these HeLa cells, the maximum change in sensitivity occurs by 2 h after the end of the first hyperthermic dose. Results to be published elsewhere demonstrate that the level of induced thermotolerance is dependent on both the temperature and the time at the elevated temperature of the first thermal dose and that it persists for about one cell cycle but is not passed on to subsequent generations. Figures 1 and 2 argue that our HeLa cells are not able to accumulate or recover from sublethal hyperthermic damage. Rather, a single hyperthermic treatment can induce a transient state of thermotolerance, but cannot produce heritable hyperthermic resistance.

We thank Mr R. Boone for technical assistance. This research was supported by the NIH and the American Cancer Society.

EUGENE W. GERNER
MICHAEL J. SCHNEIDER

University of Arizona Medical School,
Division of Radiation Oncology,
Tucson, Arizona 85724.

[1] Rao, P. N., and Engelberg, J., *Science*, 148, 1092-1904 (1965).
[2] Palzer, R. J., and Heidelberger, C., *Cancer Res.*, 33, 422-427 (1973)
[3] Dickson, J. A., and Muckle, D S., *Cancer Res*, 32, 1916-1923 (1972)
[4] Westra, A., and Dewey, W. C., *Int J. Radiat. Biol.*, 19, 467-477 (1971).
[5] Palzer, R. J., and Heidelberger, C, *Cancer Res*, 33, 415-421 (1973).
[6] Harris, M., *Expl Cell. Res*, 46, 301-314 (1967).
[7] Hahn, G, *Cancer Res.*, 34, 3117-3123 (1974).

Regional turnover and synthesis of catecholamines in rat hypothalamus

THE occurrence of catecholaminergic systems in the hypophysiotropic area of the hypothalamus has led to numerous studies of the role of catecholamines in the control of pituitary function[1-4]. The lack of biochemical methods of sufficient sensitivity to estimate catecholamine concentrations and metabolism in small brain parts has, however, hampered progress in this field. It has been claimed on the basis of histochemical fluorescence studies, that predominantly dopamine is present in the median eminence of the rat (see ref. 3). Other reports, dealing with the fluorometric determination of catecholamine concentrations, have claimed that rat median eminence dopamine levels are low relative to the noradrenaline concentrations (see ref. 3). Using sensitive radiochemical catecholamine assays, both claims were disproved[5,6]. The considerable regional differences

in catecholamine concentrations in hypothalamic nuclei observed by Palkovits *et al.*[6] stress that great care should be taken to properly dissect the hypothalamus, a conclusion also indicated by Kavanagh and Weisz[7].

We present here data on regional hypothalamic noradrenaline and dopamine metabolism obtained with two methods, one involving measurement of the rate of catecholamine loss following synthesis inhibition using a sensitive radiochemical assay, and the other involving estimation of the rate of accumulation of ^3H-catecholamines from L-^3H-tyrosine *in vitro*.

Male Wistar rats (130–140 g) were used. The hypothalamus was dissected into median eminence, the area including the part of the arcuate nucleus dorsal to the median eminence, and the residual hypothalamus[5,7,8]. The average weights of the dissected regions were 0.51, 1.92 and 32.6 mg, respectively.

In the first series of experiments rats were given α-methyl-p-tyrosine methyl ester HCl (α-MPT; 300 mg kg^{-1}, intraperitoneally) 4, 2 or 1 h before decapitation. Noradrenaline, dopamine and, for steady state levels only, adrenaline, were assayed in the hypothalamus parts of untreated and α-MPT-treated rats, using a radiochemical method derived from that described previously (ref. 9 and details in J.G., H.L.J.M. Wijnen and D.H.G.V., unpublished).

Table 1 summarises the concentrations of noradrenaline, dopamine and adrenaline in the three regions investigated, the rate constants of noradrenaline and dopamine loss after α-MPT and the turnover rates calculated from these[10]. The plots of the logarithms of catecholamine concentrations against time were linear for both amines in all three regions. The steady state levels are close to those already reported[5-7]. Although the rate constant of dopamine loss in the median eminence was somewhat lower than in the arcuate nucleus area and the residual hypothalamus, the rate of dopamine turnover in the median eminence was extremely high relative to that in the other regions. Both the rate constant of noradrenaline loss and the noradrenaline turnover rate were markedly lower in the median eminence than in the other regions. A possible explanation for this phenomenon is the occurrence of a significant amount of median eminence noradrenaline in an extraneuronal pool[11]. The turnover of noradrenaline in the median eminence was considerably slower than that of dopamine. This is in agreement with the findings of Löfström *et al.*[12], who used fluorescence histochemistry in combination with microfluorometry to measure catecholamine depletion in the median eminence after synthesis inhibition, and observed a much lower catecholamine turnover in the subependymal layer, supposedly containing mainly noradrenaline terminals, than in the external layer, which is presumed to contain predominantly dopamine terminals.

In the second series of experiments hypothalamus parts were incubated for 45 min in Krebs–Henseleit bicarbonate medium in the presence of L-3,5-^3H-tyrosine to estimate the accumulation of tritiated catecholamines from the tritiated precursor amino acid as described previously for larger brain parts[13].

As can be seen from Table 2, a gradient was observed in the *in vitro* accumulation of ^3H-dopamine from median eminence to deeper hypothalamic structures, similar to that for the dopamine turnover rates. ^3H-noradrenaline accumulation, however, was of the same order of magnitude in all three regions. This latter observation is at variance with the data obtained from the α-MPT experiments, which suggested a slower turnover of noradrenaline in the median eminence than in the arcuate nucleus area and the residual hypothalamus. This may indicate that noradrenaline synthesis in the neuronal pool in the median eminence is relatively high, and suggests one of the disadvantages of the α-MPT method, that is, that it does not allow discrimination between various amine pools in the same brain region with different turnovers. Apart from this feature the results obtained with the two methods are similar. Both methods enable discrimination to be made between noradrenaline and dopamine metabolism in very small areas of brain and can be helpful in

Table 1 Steady state level, turnover and turnover rate of noradrenaline and dopamine in rat median eminence (ME), arcuate nucleus area (ANA) and residual hypothalamus (RH)

	Tissue weight (mg)	Noradrenaline			Dopamine			Adrenaline
		0 h level (μg g^{-1})	Turnover (k) (h^{-1})	Turnover rate (μg g^{-1} h^{-1})	0 h level (μg g^{-1})	Turnover (k) (h^{-1})	Turnover rate (μg g^{-1} h^{-1})	0 h level (μg g^{-1})
ME	0.50$\pm$0.01	3.57$\pm$0.24 (6)	0.051$\pm$0.006	0.185	7.64$\pm$0.67 (5)	0.310$\pm$0.009	2.194	NM (6)
ANA	1.93$\pm$0.04	3.03$\pm$0.17 (6)	0.210$\pm$0.009	0.549	0.50$\pm$0.06 (5)	0.470$\pm$0.012	0.212	0.121$\pm$0.004 (5)
RH	32.7$\pm$0.4	2.54$\pm$0.12 (6)	0.159$\pm$0.007	0.398	0.32$\pm$0.01 (5)	0 445$\pm$0.009	0.118	0.046$\pm$0.006 (6)

Hypothalamus parts of untreated rats and of rats which had been treated with α-MPT (H44/68, AB Biotec, Stockholm; 300 mg kg^{-1} intraperitoneally) 4, 2 or 1 h before decapitation were homogenised in 50–200 volumes 0.1 N $HClO_4$. Noradrenaline, dopamine and, for steady state levels only, adrenaline were assayed in 20-μl samples of the supernatants using a radiochemical method involving the paper chromatographic separation of the ^{3}H-methoxy derivatives of the catecholamines obtained by incubation of the samples with a rat liver catechol-O-methyltransferase preparation and S-adenosyl-L-methyl-^{3}H-methionine (Radiochemical Centre, Amersham; 5–9 Ci mmol^{-1}), (J.G., H. L. J. M. Wijnen and D.H.G.V. unpublished) Blanks, tissue blanks and internal as well as external standards were run parallel with the samples. Sensitivity of the assay for all three catecholamines was approximately 15 pg. Values have been corrected for cross interference, which amounted to less than 2%. The rate constants of amine loss (k) and turnover rates of the catecholamines were calculated according to Brodie et al.[10]. Results are expressed as mean values$\pm$s.e.m.; number of observations for levels was as indicated in brackets; number of observations for tissue weights, ks and turnover rates was 22–24.

NM, Not measurable because of interference from high dopamine levels.

Table 2 In vitro accumulation of ^{3}H-noradrenaline and ^{3}H-dopamine from L-^{3}H-tyrosine in rat median eminence (ME), arcuate nucleus area (ANA) and residual hypothalamus (RH)

	Tissue weight (mg)	^{3}H-tyrosine (μCi mg^{-1})	$\dfrac{^3\text{H-noradrenaline}}{^3\text{H-tyrosine}}$	$\dfrac{^3\text{H-dopamine}}{^3\text{H-tyrosine}}$
ME	0.52$\pm$0.01	0.657$\pm$0.029	4.37$\pm$0.23	44.6$\pm$3.7
ANA	1.92$\pm$0.02	0.308$\pm$0.014	4.89$\pm$0.22	10.69$\pm$0.83
RH	32.6$\pm$0.3	0.035$\pm$0.001	4.32$\pm$0.19	6.24$\pm$0.032

ME and ANA were preincubated for 10 min at 37 °C in 0 5 ml Krebs-Henseleit bicarbonate buffer, pH 7.2; RH, chopped in 0.3 mm slices with a McIlwain Tissue Chopper, in 2.0 ml of this buffer. Subsequently 50 μl buffer containing L-3,5-^{3}H-tyrosine (Radiochemical Centre, Amersham) of appropriate specific activity were added and incubation was continued for 45 min. The specific activity of the ^{3}H-tyrosine added was 21.0 Ci mmol^{-1} (ME), 9.8 Ci mmol^{-1} (ANA) or 1.16 Ci mmol^{-1} (RH); the final concentration of tyrosine in all incubation media was 8.6 μM. The incubation was stopped by addition of 4.0 ml ice-cold buffer. After centrifugation and a wash cycle the pellets were homogenised in 0.4 N $HClO_4$. Carrier tyrosine, noradrenaline and dopamine were added and ^{3}H-tyrosine, ^{3}H-noradrenaline and ^{3}H-dopamine were purified on Dowex 50WX4 and alumina columns as described previously[13]. Tissue medium ratios for ^{3}H-tyrosine were close to 3 6 in all cases. Accumulation of tritiated catecholamines from ^{3}H-tyrosine was linear for at least 45 min, and seemed to be directly proportional to the specific activity of the tyrosine added to the incubation medium. Thus, the ratio of ^{3}H-catecholamines (nCi per mg tissue) to ^{3}H-tyrosine (μCi per mg tissue) was taken as an index of ^{3}H-catecholamine synthesis. Results are expressed as mean values$\pm$s.e.m. Number of observations was 24–27.

studies dealing with pharmacological and endocrinological manipulation and catecholamine activity in localised brain structures.

We thank Mrs W. Spaapen-Kok and Mr H. Spierenburg for assistance. This study was partly supported by the Foundation for Medical Research (FUNGO), which is subsidised by the Netherlands Organisation for the Advancement of Pure Research (ZWO).

DIRK H. G. VERSTEEG
JAN VAN DER GUGTEN
JAN M. VAN REE

Rudolf Magnus Institute for Pharmacology,
Medical Faculty, University of Utrecht,
Vondellaan 6, Utrecht, The Netherlands

1 Hokfelt, T , and Fuxe, K , in Brain–Endocrine Interaction, Median Eminence Structure and Function, 181–223 (Karger, Basel, 1972)
2 de Wied, D., and de Jong, W , A. Rev. Pharmac., 14, 389–412 (1974).
3 Bjorklund, A , Falck, B , Nobin, A , and Steveni, U , in Neurosecretion—The Final Neuroendocrine Pathway (edit. by Knowles, F., and Vollrath, L), 209–222 (Springer, Berlin, 1974).
4 Versteeg, D H. G., and Wurtman, R. J , in Chemical Neurobiology (edit. by Gispen, W H), (ASP Biological and Medical, Elsevier Division, Amsterdam, in the press)
5 Cuello, A C , Horn, A. S , Mackay, A. V P., and Iversen, L L , Nature, 243, 465–467 (1974)
6 Palkovits, M., Brownstein, M., Saavedra, J M , and Axelrod, J , Brain Res., 77, 137–149 (1974)
7 Kavanagh, A , and Weisz, J , Neuroendocrinology, 13, 201–212 (1973/74).
8 Gispen, W H., Schotman, P , and de Kloet, E. R , Neuroendocrinology, 9, 285–296 (1972)
9 Cuello, A C., Hiley, R , and Iversen, L L , J Neurochem., 21, 1337–1340 (1973)
10 Brodie, B B., Costa, E , Diabac, A., Neff, N. H., and Smookler, H H , J. Pharmac exp Ther., 154, 493–498 (1966)
11 Knigge, K. M , in Frontiers in Neurology and Neurosciences Research (edit. by Seeman, P., and Brown, G M), 40–47 (Neuroscience Institute, University of Toronto, 1974)
12 Fuxe, K., Hokfelt, T., Jonsson, G., and Lofstrom, A , in Neurosecretion—The Final Neuroendocrine Pathway (edit. by Knowles, F., and Vollrath, L.), 269–276 (Springer, Berlin, 1974)
13 Versteeg, D. H. G., van Ree, J. M., Provoost, A. P., and de Jong, W , Life Sci., 15, 2127–2134 (1974)

Rate of nucleologenesis as a measure of gene activity

THE nucleolus is not a permanent organelle in the life of a cell; at the initiation of a new cycle, the nucleolar formation seems to depend on RNA synthesis directed by nucleolar RNA polymerase[1]. We have compared the rate of nucleologenesis in meristem cells of Allium cepa L. and in those of four Vicia species with different numbers of ribosomal cistrons and different DNA contents[3,4] as well as the rate of nucleologenesis in four different cell populations lying side by side in the root of Zea mays. The rate of nucleologenesis per ribosomal cistron seems to be constant in the different species growing in similar conditions, but this mean rate was modified in metabolically different subpopulations in the same root.

We used primary root meristems of V. faba, V. narbonensis, V. sativa, V. villosa and A. cepa growing at 15 °C in constantly aerated water in the dark and Zea mays roots growing at 23 °C in damp sphagnum, similarly in the dark (see Fig. 1). Roots (2–4 cm long) were treated for 1 h with 0.1 % caffeine, which inhibits cell plate formation—cytokinesis—in those cells undergoing (during treatment) the final stages of mitosis and thus marked as binucleate at the start of a new cycle.

Telophase ⟶ Binucleate cell (new cycle)
Caffeine
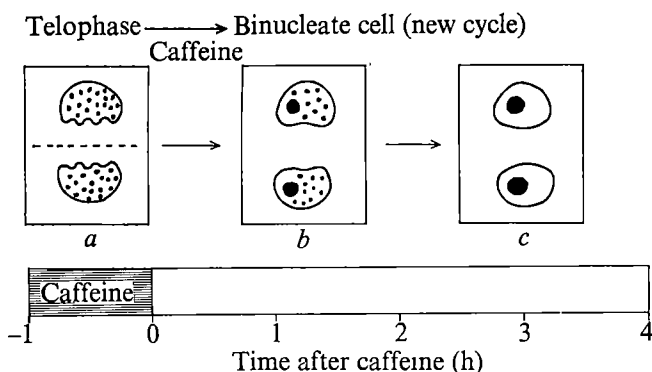
Fig. 1 Experimental scheme. Caffeine labels those cells going through telophase during treatment Recording of the binucleate cells with fully organised nucleoli at successive hours after treatment provides a direct measure of the rate of nucleologenesis in these cells. *a*, Prenucleolar bodies in telophase; *b*, prenucleolar bodies and incipient nucleoli in early G_1; *c*, fully organised nucleoli.

The synchrony of these binucleate cells is excellent, particularly in the short term; and as nucleologenesis takes place precisely at those stages of the cell cycle which coincide with, or occur immediately after, caffeine labelling, these binucleate cells make suitable subjects to study the kinetics of nucleologenesis.

Figure 2 shows the appearance of fully organised nucleoli in the four *Vicia* species. The time at which 50% of the binucleate cells had fully organised nucleoli was estimated and the final nucleolar volumes on completion of nucleologenesis were scored (Table 1). In all species studied the maximum number of nucleoli was two, indicative of species with one pair of nucleolar organiser chromosomes. In 50% of cells these two nucleoli fused into one nucleolar mass. In *V. sativa*, however, only 3% of the nuclei showed two nucleolar masses.

We have estimated the rate of nucleologenesis per ribosomal cistron (Table 2), and found that in meristems of the four species of *Vicia* and in *A. cepa*, it is practically constant, with an average value of $2.0 \pm 0.2 \times 10^{-3} \, \mu m^{-3} \, h^{-1}$ per cistron.

The rate of nucleologenesis in stele 1 of maize roots is

Table 1 Nucleolar parameters

	Time of nucleologenesis* (h)	Nucleolar volume† (μm^3; mean ± s.e)
V. villosa	4.7	26.3±2.5
V. sativa	3.7	24.6±2.3
V. narbonensis	2.9	36.0±3.1
V. faba	3.8	79 1±6.8
A. cepa	1.6	43.6±4 0
Zea mays		
Cap initials	0.3	7.8±0.6
Quiescent centre‡	1 1	5.4§
Stele 1	0.7	8.2±0.7
Stele 2	0.5	9.8±0.8

*Time when 50% of the binucleate cells have fully organised nucleoli.

†Volumes were measured the first full hour after the recorded timing of nucleologenesis (that is, in the fifth hour after caffeine in *V. villosa*; in the fourth hour in *V. sativa*, and so on).

Vicia and *Allium* roots were silver impregnated according to the scheme of Fernández-Gómez *et al.*[14]. This impregnation preferentially contrasts protein which accumulates in the fibrillar moieties of nucleoli and, to a lesser extent, in the granular zones[15] It gives a very good nucleolar image. Squashes were prepared of the terminal 2 mm of the root, where a fairly pure meristematic zone is found. *Zea* roots were silver impregnated as above [and, later dehydrated, embedded in paraffin, and sectioned at 5 μm. Median sections of 40 roots were scored. Stele 1 corresponds to steler cells just above the quiescent centre and stele 2 to a zone 200 μm further away.

‡Because of the small size of this population and its low proliferation rate, the timing of nucleologenesis has been taken from previous data, taking into account the frequency of cells with reorganising nucleoli and the cycle duration[16].

§Volume corresponds to mean nucleolar volume in the zone.

similar to that shown by the meristems of *Vicia* and *Allium*, whereas the rate of nucleologenesis in a stelar zone more remote from the quiescent centre (stele 2) is higher. An intermediate value between those found in steles 1 and 2 would probably be representative of total meristematic behaviour. According to Table 2 this intermediate value would be about $2.5 \times 10^{-3} \, \mu m^{-3} \, h^{-1}$ per cistron, that is, higher than that obtained for *Vicia* and *Allium*. This may be attributable to different growing temperatures (23 °C for maize and 15 °C for *Vicia* and *Allium*), as temperature may be an important factor in determining the duration of nucleologenesis—it certainly is in determining the duration of the cycle[6].

The fact that the nucleologenesis rate is similar in the root meristems of at least five different species could mean that all the ribosomal cistrons are operational and transcribed at a similar rate during this process. The mean rate per cistron, however, does not correspond to the optimum, as in cap initial cells in maize a rate of $4.2 \times 10^{-3} \, \mu m^{-3} \, h^{-1}$ per cistron was measured, that is, twice the average value obtained in complete meristems. Similarly, in the case of wild type *Xenopus*, 'normal' transcriptional activity is not the optimum, as studies[12,13] of rRNA synthesis in *Xenopus* mutants deficient in ribosomal cistrons have shown that a normal transcriptional rate could be achieved with half the wild type number of cistrons.

The study of nucleologenesis in *A. cepa* in the presence of metabolic inhibitors showed that inhibition of nucleolar transcription impedes nucleologenesis. Surprisingly, protein synthesis inhibition accelerates nucleologenesis[1,9]. Thus, nucleologenesis may be modulated by concurrent metabolic processes in the cell. In this respect, Clowes[6,7] and Barlow[8] have shown very different rates of incorporation of radioactive precursor into proteins and RNA between the maize populations studied. This could explain the divergence from the 'normal' rate in the quiescent centre and cap initials.

Significant differences in nucleolar transcriptional activity of ribosomal cistrons exist, for example, between pre- and postgastrula stages in *Xenopus*[2], and in mutants, even though this is not the case for other transcriptional modulations. Thus, Mukherjee and Beermann[10] demonstrated that in *Drosophila* there was dosage compensation for the transcriptional rate in the single X chromosome in males compared with the two X chromosomes in females. Similarly Ananiev *et al.*[11] studied the transcriptional rate in *Drosophila* genotypes differing in the proportion of X chromosomes to autosomes and showed that the dosage compensation of enzyme synthesis coded by sex-linked genes took place during transcription.

Fig. 2 Percentage of binucleate cells with fully organised nucleoli at successive hours after caffeine treatment. Time when 50% of these cells had completed nucleologenesis may be obtained from this graph. △, *V narbonensis*; ▲, *V. faba*; ●, *V. sativa*; ○, *V villosa*.
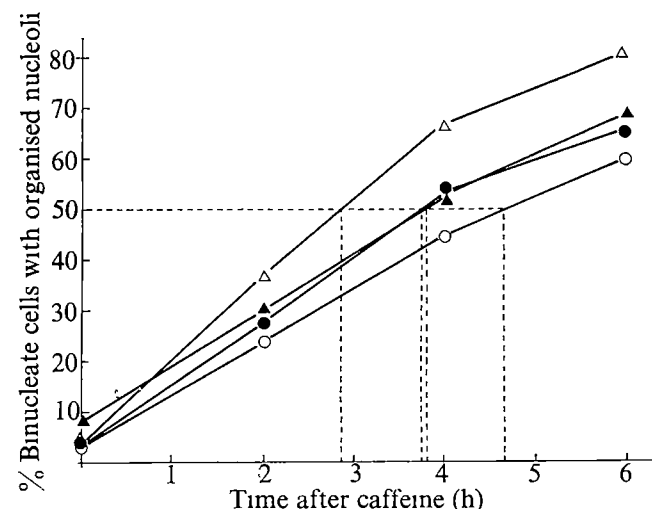
Table 2 Number of ribosomal cistrons and rate of nucleologenesis

	DNA content in 2C nuclei (pg)*	No. of ribosomal cistrons ($\times 10^{-3}$)†	Nucleologenesis rate ($\times 10^{-3}$ μm^{-3} h^{-1} per cistron)
V. villosa	3.83	2.50	2.2
V. sativa	3.92	3.75	1.8
V. narbonensis	14.38	6.25	2.0
V. faba	29.56	9.50	2.2
A. cepa	33.00	13.30‡	2.0
Zea mays	7.73	6.2‡	—
Cap initial	—	—	4.2
Quiescent centre	—	—	0.7
Stele 1	—	—	1.9
Stele 2	—	—	3.2

*DNA content was estimated by microdensitometry on Feulgen-stained nuclei (in a Vickers scanning microdensitometer). The value of 33 pg in 2C A. cepa nuclei were used for the conversion into the absolute values.
†Ref. 4.
‡Ref. 3.

We postulate that the rate of nucleologenesis may be used as a direct test of the activity of a gene. The possibility of manipulating gene action and quantifying the response modulation of ribosomal cistrons in this test remains open.

This work was supported by the Plan de Desarrollo, Spain, and by a Rodríguez Pascual grant.

C. DE LA TORRE
M. E. FERNANDEZ-GOMEZ
G. GIMENEZ-MARTIN

Departamento de Citología,
Instituto de Biología Celular (CSIC),
Velázquez, 144, Madrid-6, Spain

1 Giménez-Martín, G., de la Torre, C., Fernández-Gómez, M. E., and González-Fernández, A., J. Cell Biol., 60, 502–507 (1974).
2 Landesman, R., Cell Different., 1, 209–213 (1972).
3 Ingle, J., and Sinclair, J., Nature, 235, 30–32 (1972).
4 Maher, E. P., and Fox, D. P., Nature, 245, 170–172 (1973).
5 López-Sáez, J. F., Giménez-Martín, G., and González-Fernández, A., Z. Zellforsch., 75, 591–600 (1966).
6 Clowes, F. A. L., J. exp. Bot., 7, 307–312 (1956).
7 Clowes, F. A. L., J. exp. Bot., 9, 229–238 (1958).
8 Barlow, P. W., J. exp. Bot., 21, 292–299 (1970).
9 Stockert, J. C., Fernández-Gómez, M. E., Giménez-Martin, G., and López-Sáez, J. F., Protoplasma, 69, 265–278 (1970).
10 Mukherjee, A. S., and Beermann, W., Nature, 207, 785–786 (1965).
11 Ananiev, E. V., Faizullin, L. Z., and Grozdev, V. A., Chromosoma, 45, 193–201 (1974).
12 Miller, L., and Gurdon, J. B., Nature, 227, 1108–1110 (1970).
13 Knowland, J. S., and Miller, L., J. molec. Biol., 53, 321–338 (1970).
14 Fernández-Gómez, M. E., Stockert, J. C., López-Sáez, J. F., and Giménez-Martín, G., Stain Techn., 44, 48–49 (1969).
15 Risueño, M. C., Fernández-Gómez, M. E., and Giménez-Martín, G., Mikroskopie, 29, 292–298 (1973).
16 de la Torre, C., and Clowes, F. A. L., J. Cell Sci., 11, 713–721 (1972).

Fig. 1 Schematic representation of 5S RNA secondary structure. Normal base pairs are connected by vertical lines, whereas G–U pairs are indicated by dots. The shaded portion of the molecule is that which has been identified as being involved in an interaction with specific 50S subunit proteins[19,20]. The structure is drawn with helices aligned along the long axis in accord with the suggestion of Connors and Beeman[30].

5S RNA secondary structure

NUMEROUS attempts have been made to deduce a secondary structure of 5S RNA[1-4]. Differing approaches have yielded various structures, but each turns out to be inconsistent with either physical, chemical, biochemical, or comparative evidence. Clearly the situation is not analogous to that which obtained for transfer RNA (tRNA). There is, however, at least one fundamental difference between the two molecules. The functional 5S RNA molecule is at all times an integral part of the 50S ribosomal subunit, whereas tRNA is a transient inhabitant of the ribosome milieu. Thus, it is possible that functional conformations of the 5S RNA molecule need only exist in the context of the ribosome, and consequently, may only exist therein. Thus, the interpretation of experimental investigations on the isolated 5S RNA molecule may have inherent difficulties.

A comparative analysis[5] of 5S RNA primary structure is one approach to defining functionally significant secondary (and tertiary) structure whose interpretation is not open to the above

caveat. A phylogenetically broad range of prokaryotic 5S RNAs have been shown to be functionally equivalent (that is, interchangeable)[6,7]. Thus, phylogenetic conservation of functionally significant features of the 5S RNA molecule is very likely. By including in such a study 5S RNAs isolated from organisms which occupy unusual ecological niches (extremes of temperature, pH, ionic strength, pressure or antibiotic tolerance), it should be possible not only to identify the normal constraints on the molecule's primary structure, but also to demonstrate how these constraints change under selective pressure. This in turn should provide clues to the functional significance of the constraints. A theoretical analysis of possible base-pairing schemes has been combined with an experimental programme of sequence determination to reveal the principal features of the architecture of this molecule and to provide insight into its mode of function[8,9].

It is the fundamental premise of this comparative approach that functionally equivalent molecules will exhibit the same secondary structure. Thus, if a helix is found in one primary sequence, it must be present in all other examples to be considered real. In addition, a base-paired region has been assumed to conform to a set of rules which are suggested by examination of tRNA secondary structures: only G–C, A–U and G–U base pairs are allowed; all helices consist of at least three base pairs without G–U pairs or four base pairs when G–U pairs are allowed; looped-out bases are assumed to be non-existent or very rare; and hairpin loops are assumed to contain at least three nucleotides.

Applying these rules, the diagonal method of Tinoco et al.[10] is used to predict all possible helices in any 5S ribosomal RNA (rRNA) molecule. Once a given helix is found to be universal, it greatly restricts the remaining helices which might exist. In the case of Escherichia coli 5S rRNA, 110 possible helices exist, and all but nine of these are ruled out by the secondary structure proposed here. None of these nine, including the largest (four base pairs, 25–29 with 104–107) are, however, universal. Thus, the structure proposed here is in this sense complete.

The general prokaryotic 5S RNA (Figs 1 and 2) contains four helices, herein referred to as the molecular stalk, the prokaryotic loop, the tuned helix, and the common arm base.

The molecular stalk is in all cases a contiguous stretch of base pairs (usually 10 or 11) which serves to tie together the two ends of the molecule[11]. The primary structure of this region is rather variable and often contains G–U pairs, for example, the three contiguous G–U pairs in the stalk of Bacillus megaterium 5S RNA[8].

The prokaryotic loop, E. coli positions 82–86 and 90–94, has been seen only in prokaryotic 5S RNAs; it comprises four to five base pairs (E. coli can be extended to eight when G–U pairs are included) and generally closes a tight hairpin of three pyrimidines or four unspecified nucleotides. Although its primary structure is somewhat variable, it contains a high

```
                      10                    20      30              40            50
E. coli              p |UGCCUGGCGG|  CCGUAGC  |GCGGUG||GUCC[CAC| CUGA |CCCCAUGCCGAAC| |UCAG||AAGUGAAA]
Photo. 8265          p |UGCUUGGCGA|  CCAUAGC  |GUUAUG||GACC[CAC| CUGA |UCCCUUGCCGAAC| |UCAG||UAGUGAAA]
Pseudo. fluor.       p |UGUUCUUUG |  ACGAGUAGUA |GCAUUG||GAA [CAC| CUGA |UCCCAUCCCGAAC| |UCAG||AGGUGAAA]
B. megaterium        p |UCUGGUGGCG|  AUAGC    |GAAGAG||GUCA[CAC| CCGU |UCCCAUACCGAAC| |ACGG||AAGUUAAG]
B. stearothermophilus p |CCUAGUGACAA| UAGCGA   |G·AGAG||GAAA[CAC| CCGU |UCCCAUCCCGAAC| |ACGG||AAGUUAAG]
A. nidulans          pU |CCUGGUGUC |  UAUGGC   |GGUAUG||GAAC[CACU| CUGA |CCCCAUCCCGAAC| |UCAG||UUGUGAAA]


Chlorella            ppp |AUGCUACGUU| CAUA·C   |ACCACG||AAAG[CAC| CCGA |UCCCAU CAGAAC| |UCGG||AAGUUAAA]
KB cell              ppp |GUCUACGGC |  CAUACC   |ACCCUG||AACG[CGC| CCGA |UCUCGU CUGAUC| |UCGG||AAGCUAAG]
X. laevis            ppp |GCCUACGGC |  CACACC   |ACCCUG||AAAG[UGC| CCGA |UCUCGU CUGAUC| |UCGG||AAGCCAAG]
T. utilis            pp |GGUUGCGGC |  CAUAUC   |UAGCAG||AAAG[CAC| |C·GUU| |CUCCGU CCGAUC| |AACUG||UAGUUAAG]
                         A                        B        C                            C'
```

```
                      60        70       80          90        100           110      120
E. coli              |CGCCGU| AG[CGCCGAUGGUAGU] G |UGGGG| UCU |CCCCA| U GCGAGAGUAGGGAA |CUGCCAGGCA| U_OH
Photo. 8265          |CGUAAU| AG[CGCCGAUGGUAGU] G |UGGGG| UCU |CCCCA| U GUGAGAGUAGGACA |UCGCCAGGCA| U_OH
Pseudo. fluor.       |CGAUGC| AU[CGCCGAUGGUAGU] G |UGGGG| UUU |CCCCA| U GUCAAGAUCUCGAC |CAUAGAGCA | U_OH
B. megaterium        |CUCUUU| AG[CGCCAAUGGUAGU]U |GGGAC| UUU |GUCCC| U GUGAGAGUAGGA   |CGUUGCCAGG| C_OH
B. stearothermophilus |CUCUCC| CAG[CGCCGAUGGUAGU]U |GGGGC| CAGC |GCCCC| U GCAAGAGUAGG   |UUGUCGCUAGG| C_OH
A. nidulans          |CAUACC| UG CGGCAACGAUAGC UC |CCGG| GUAG |CCGG| UCGCUAAAAUAGCUC  |GACGCCAGG | UC_OH
                                                    D         D'

Chlorella            |CGUGGU| UGGG CUCGA  CUAGUACU(GGGUU)GGAGGAUUACCUGAGUGGG(AACCC )C |GACGUAGUGU| _OH
KB cell              |CAGGGU| CGGG(CCUGGU) UAGUACU UGGAU GGGAGACCGCCUGGGAAU (ACCGGG)U |GCUGUAGGC| U(U)U_OH
X. laevis            |CAGGGU| CGGG(CCUGGU) UAGUACU UGGAU GGGAGACCGCCUGGGAAU (ACCAGG)U |GUCGUAGGC| UUU_OH
T. utilis            |CUGCUA| AGAG(CCUGA)UCGAGUAGU GUAGU GGGUGACCAUACGCGAAAC(UCAGG )U |GCUGCAAUC| U_OH
                         B'                                                               A'
```

Fig. 2 Ten known sequences of the 5S RNA molecule which differ substantially are divided into two groups—corresponding to prokaryotes and eukaryotes[1,8,9,11,17,18,31–35]. The alignment is adjusted to juxtapose secondary structural features. Vertical lines indicate regions of base pairing and brackets enclose regions of high phylogenetic conservation. Parentheses indicate regions of base pairing in the 3' half of the eukaryotic molecule[1,17]. The sequences are as published elsewhere with the exception of *B. stearothermophilus*, the unsequenced portions of which are arranged in a manner consistent with the proposed structure. In this case also, a correction is included—the oligomer UCUCCAUCCCG is replaced by UUCCCAUCCCG. Position numbers in the *E. coli* sequence are included as subscripts, as used in the text.

portion of G–C pairs in those cases so far examined; and in the case of *E. coli*, has been reported to be rather resistant to nuclease attack[11]. This loop, too, has been included in many previous models, although a possible alternative pairing between *E. coli* positions 33–42 and 79–88 has frequently been suggested[12]. Comparative study makes the latter unlikely (as both the precise location and length of the pairing would have to

vary considerably). Until this alternative can be eliminated, however, the proposed structure cannot be claimed to be unique.

The third helix, *E. coli* positions 18–23 and 60–65, is referred to as 'tuned' because its thermodynamic stability as estimated by the method of Tinoco *et al.*[13] seems to be under energetic constraints. This constraint is quite unique to this helix and varies from extreme weakness in marine prokaryotes (four

examined so far), to intermediate values in non-marine pro-karyotes, and to quite stable structure in all eukaryotes[9]. As the total length of this helix, six base pairs, is highly conserved (*B. stearothermophilus* represents either an exception or a sequencing problem), the energetic control is obtained by significant variations in composition. The terminal G–C pair (*E. coli* positions 23 and 60) is, however, found in all organisms examined so far.

Finally, the common arm base, *E. coli* positions 31–34 and 48–51, is so termed because it closes a 12–13 base loop which contains in all prokaryotes examined so far the sequence CGAAC, which reportedly interacts with the tRNA common arm sequence GTΨCG (refs 14–16). The common arm base comprises four base pairs in all cases except yeast, which seems to contain only three [17,18]. If the loop closed by this helix does interact with the common arm of tRNA, a contiguous stretch of at least eight base pairs could result (four within the 5S RNA and four or five between the tRNA and 5S RNA). The effect of the formation of such an intermolecular helix on the tRNA common arm geometry and thus overall tRNA tertiary structure would be quite drastic.

Whereas primary structure is not generally highly conserved in the four double-stranded regions, two single-stranded regions in the molecule are, in fact, strongly conserved. The smaller of these, GCGCC^G_AAUGGUAGU (*E. coli.* positions 67–80), is found in that region of the molecule protected by three 50S ribosomal proteins (see Fig. 1)[19,20]. This sequence is found in all published bacterial 5S RNA structures, but is not entirely universal, being absent in the eukaryotes and *Anacystis*. The second, larger region of high conservation, positions 28–59 in *E. coli*, includes the common arm base and, near its middle, the universal prokaryotic sequence CGAAC. The molecule (Fig. 1) can thus be crudely pictured as consisting of two regions, both containing strongly conserved sequences; one of recognised functional importance (5S–tRNA interaction), the other apparently of structural importance (perhaps 5S interaction with the 50S particle). Separating these two large regions is the tuned helix.

Two additional general constraints which have been observed in the nine existing 5S RNA sequences are: the first four nucleotides in the loop defined by the common arm base, although variable, are in all cases pyrimidines (four different examples thus far); and a stretch of at least four purines exists about twenty nucleotides from the 3′ end. Pace *et al.* have suggested the latter to be a recognition signal for a posttranscriptional modification enzyme in *B. subtilis*[21,22].

The proposed model for prokaryotic 5S RNA secondary structure is quite consistent with the comparative evidence. Each helix has been substantiated by examples wherein significant base changes in its primary structure have occurred without violating the proposed base-pairing constraint. In addition, it is in agreement with most of the evidence obtained by physical and chemical methods. In particular, the recent high resolution nuclear magnetic resonance prediction of 28 base pairs, including four A–U in *E. coli*, is in excellent agreement with the proposed structure, that is, 25 base pairs, five of which are A–U. Chemical modification studies using a variety of reagents yield no examples of definitively placed modified nucleotide residues that occur in proposed double-stranded regions.

Oligonucleotide binding studies[23], however, suggest that in *E. coli* regions 9–10 and 59–65—both involving proposed helices—can pair to external tri- and tetranucleotides. The interpretation that these regions are, therefore, not base paired in the intact molecule is based on the assumption that external tri- and tetranucleotides cannot perturb secondary structure. This assumption seems questionable in the former instance at least where the final two base pairs of the molecular stalk (one a G–U pair) would be replaced by a tetramer pairing that not only stacks on the remaining (eight) pairs of the stalk, but consists itself of four G–C pairs. Also, if Lewis and Doty[24] were not working with the native or A form of 5S RNA, their

conclusion regarding region 59–65 would not apply to that form.

The results of Vigne and Jordan[25] are also inconsistent with the present model only if one assumes that two or more breaks in the molecular backbone never disrupt secondary structure, which again is highly questionable.

Although the eukaryotic 5S RNA molecule is quite similar[1], it is distinctly different from its prokaryotic counterpart in that it lacks the prokaryotic loop and the universal sequence CGAAC. This is highly suggestive of a subtle divergence in the mechanism of protein synthesis which has been consistently hinted at in other comparisons of eukaryotic and prokaryotic ribosomes[26].

We feel that the tuned helix has a crucial role in 5S RNA structure and, consequently, function. Perhaps the most convincing evidence of this comes from studies involving the 'native' (and closely related A form) against the B form of this molecule[24,27]. There are specific points of chemical modification characteristic of each form[28]. Similarly, defined points readily accessible to enzymatic attack characterise each form[29]. One notable difference between the two forms is the accessibility to attack of bases in the tuned helix; that is, in the native form they seem to be paired, whereas they seem to be uncoiled in the B form. Although there is at present no evidence that these two forms are functionally significant, it does seem clear that the tuned helix is thermodynamically sensitive and is a major determinant in 5S RNA conformation.

This observation, the apparent energetic constraint, and the central position of the tuned helix suggest that the coiling and uncoiling of that structure is a key element in 5S RNA function. As the tuned helix could control the position of the common arm base which it encloses, it could therefore control the position and(or) conformation of the tRNA molecule. Thus, this coiling/uncoiling event would make possible a cyclic (and possibly time-symmetric) interaction of the 5S RNA with the two tRNA molecules during protein synthesis.

GEORGE E. FOX
CARL R. WOESE

Provisional Department of Genetics and Development,
University of Illinois,
Urbana, Illinois 61801

1 Jordan, B R , Galling, G , and Jourdan, R., *J. molec Biol.*, 87, 205–225 (1974).
2 Kearns, D R , and Wong, Y. P., *J molec. Biol*, 87, 755–774 (1974)
3 Lewis, J. B., and Doty, P., *Nature*, 255, 510–512 (1970).
4 Cantor, C R., *Proc natn Acad. Sci U S A*, 59, 478–483 (1968)
5 Richards, E. G , *Eur. J Biochem*, 10, 36–42 (1969).
6 Wrede, P , and Erdmann, *FEBS Lett.*, 33, 315–319 (1973)
7 Bellemare, G., Vigne, R , and Jordan, B. R , *Biochimie*, 55, 29–35 (1973)
8 Pribula, C. D , Fox, G. E., Woese, C R , Sogin, M , and Pace, N. R , *FEBS Lett.*, 44, 322–323 (1974)
9 Woese, C. R., Pribula, C. D , Fox, G E., and Zablen, L , *J molec Evol*, 5 (in the press).
10 Tinoco, I , Jr, Uhlenbeck, O C., and Levine, M. D., *Nature*, 230, 362–367 (1971)
11 Brownlee, G. G , Sanger, F , and Barrell, B. G , *J. molec Biol*, 34, 379–412 (1968).
12 Siddiqui, M. A Q , *J Sci. Ind Res*, 32, 34–44 (1973)
13 Tinoco, I , Jr, *et al*, *Nature new Biol.*, 246, 40–41 (1973)
14 Erdmann, V A , Sprinzl, M., and Pongs, O., *Biochem. biophys. Res Commun.*, 54, 942–948 (1973).
15 Dube, S K , *FEBS Lett.*, 36, 39–42 (1973)
16 Richter, D , Erdmann, V. A , and Sprinzl, M , *Nature new Biol.*, 246, 132–135 (1973)
17 Nishikawa, K , and Takemura, S , *FEBS Lett.*, 40, 106–109 (1974).
18 Hindley, J , and Page, S. M., *FEBS Lett*, 26, 157–160 (1972)
19 Gray, P N , Bellemare, G , and Monier, R , *FEBS Lett*, 24, 156–159 (1972)
20 Gray, P N., Bellemare, G , Monier, R., Garrett, R. A., and Stoffler, G., *J molec Biol*, 77, 133–151 (1973)
21 Sogin, M L , and Pace, N R , *Nature*, 252, 598–600 (1974).
22 Pace, N R., Pato, M. L , McKibbin, J , and Radcliffe, C. W., *J. molec Biol*, 75, 619–631 (1973).
23 Lewis, J B , and Doty, P., *Nature*, 225, 510–512 (1970).
24 Aubert, M , Scott, J F , Reynier, M., and Monier, R , *Biochemistry*, 61, 292–299 (1968)
25 Vigne, R , and Jordan, E. R , *Biochimie*, 53, 981–986 (1971)
26 Haselkorn, R , and Rothman-Denes, L B , *A Rev. Biochem.*, 42, 397–438 (1973)
27 Mirzabekov, A D , and Griffen, B E , *J molec. Biol*, 72, 633–643 (1973).
28 Aubert, M., Bellemare, G , and Monier, R , *Biochimie*, 55, 135–142 (1973).
29 Jordan, B R., *J molec Biol*, 55, 423–439 (1971).
30 Connors, P G , and Beeman, W. W., *J. molec. Biol*, 71, 31–37 (1972).
31 Forget, B. G , and Weissman, S M., *J biol Chem.*, 244, 3148–3165 (1969)
32 Dubuy, B , and Weissman, S M , *J. biol. Chem*, 246, 747–761 (1971)
33 Brownlee, G. G , Cartwright, E , McShane, T , and Williamson, R , *FEBS Lett*, 25, 8–20 (1972).
34 Marotta, C A , Levy, C. C , Weissman, S M., and Varricchio, F., *Biochemistry.*, 12, 2901–2904 (1973).
35 Cory, M J , Payne, P I., and Dyer, T. A , *FEBS Lett.*, 46, 63–66 (1974)

Differential effect of plasma fractions from normal and tumour-bearing rats on nuclear RNA restriction

WE have already presented preliminary evidence[1] that crude plasma from tumour-bearing rats contains elevated concentrations of macromolecules which stimulate the release of messenger RNA (mRNA) from isolated nuclei. The detection of these putative regulatory components was facilitated by a cell-free system developed originally to study messenger[2-5] and ribosomal[6,7] RNA (rRNA) processing and transport *in vitro*. Since both maximal RNA transport and normal nuclear RNA restriction *in vitro* (that is, equivalence between messengers released from nuclei *in vivo* and *in vitro*) require the presence of macromolecules from homologous cytosol[2,8] it is possible that the tumour cell cytoplasm is the source of the putative regulatory macromolecules in the peripheral blood of tumour-bearing animals. This possibility is supported by evidence[9,10] that factors involved in the regulation of cellular proliferation are released to circulation from tumour cells, and by the observation[11] that the enzyme composition of the host liver of tumour-bearing rats converges to that of the tumour. The spectrum of messengers transported from normal rat liver nuclei in homologous cytosol is significantly different from that transported in hepatoma cytosol[3], suggesting that differences exist in these putative regulatory components of the two tissues.

This study evaluates the relative RNA transport activity of the plasma from normal and tumour-bearing rats following partial purification of the putative regulatory components on a DEAE-cellulose column and attempts to rationalise their increase in tumour-bearing animals. Sixfold higher transport activity of a plasma fraction from tumour-bearing rats is relevant to the problem of the host–tumour relationship and emphasises the potential usefulness of this assay in diagnosis. The differences in the RNA sequences transported in response to plasma fractions from normal and tumour-bearing rats is compatible with the theory that some originate from the tumour cells.

Heparinised plasma from normal rats of the Sprague–Dawley strain, or from rats bearing the Novikoff ascites hepatoma intraperitoneally, or the Morris hepatomas 9618A or 5123D intramuscularly in the hind legs[12], was passed through a Sephadex G-25 column before testing or fractionation on DEAE-cellulose. The cell-free test system contained normal rat liver nuclei (prelabelled *in vivo* for 30 min with 6-^{14}C-orotic acid) in homologous cytosol fortified with components (see Fig. 1). The concentration of the components were identical to the standard nuclear restriction assay[3,4] with the exception of cytosol protein, which was reduced from a concentration of 12.0 to 4.0 mg ml^{-1}; this modification increased the sensitivity of the assay to exogenous sources of putative regulatory components, yet was sufficient to maintain both an effective concentration of ribonuclease inhibitor and the integrity of the nuclei[7]. The labelled RNA transported in 30 min at 30 °C, the bulk of which is messenger[2-5], was precipitated from the nuclei-freed supernatants in the presence of carrier yeast RNA with 5% trichloroacetic acid, washed with 95% ethanol and assayed in liquid scintillant. The sequence homology of the transported messengers was determined by competitive RNA–DNA hybridisation as described previously[3,4,8]. Shown in Fig. 1*a* is the DEAE–cellulose elution profile of protein (A_{280}) in plasma from normal rats (controls) and rats bearing the Morris hepatoma 5123D (approximately 1.0 cm in diameter; bilateral), which has a transplant generation time of 1 month. The plasma elution profiles (not shown) of rats bearing the Morris hepatoma 9618A (approximately 1.0 cm diameter; bilateral) or the rapidly growing Novikoff
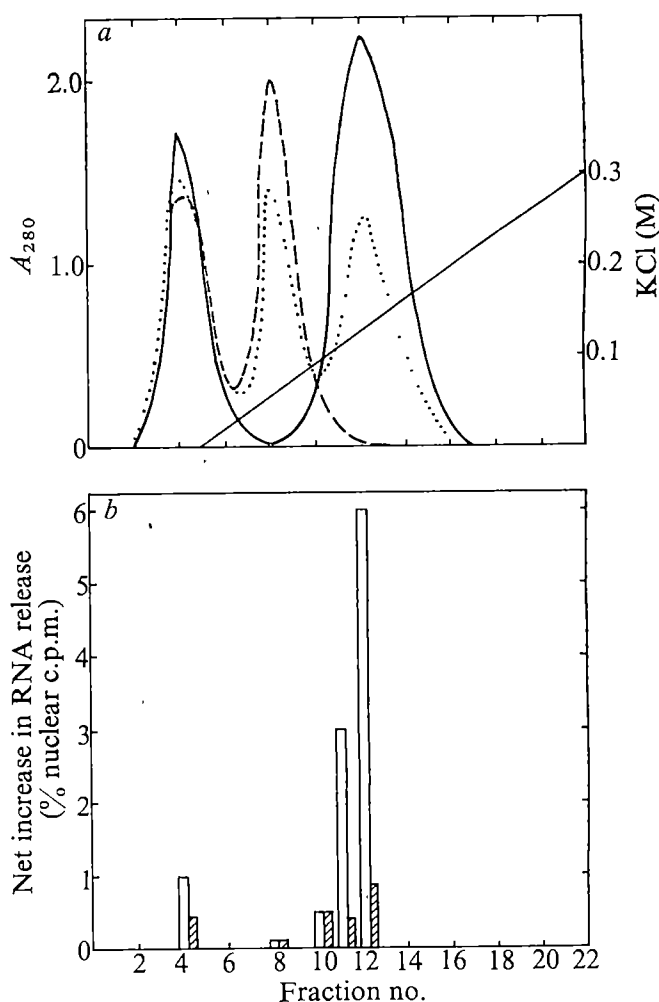
Fig. 1 *a*, DEAE–cellulose elution (A_{260}) profiles of plasma proteins from normal rats (- - -), bearing an 18-d (non-palpable) hepatoma 5123D (. . . .) and rats bearing a large (1.0 cm) hepatoma 5123D (———). In each case, the equivalent of 1.0 ml of plasma was passed through a Sephadex G-25 column and the proteins were adsorbed on a DEAE-cellulose column (1.0 × 20 cm) equilibrated with 0 02 M K$_2$HPO$_4$ (pH 7 4); the adsorbed protein was eluted with a linear (0.0–0.5 M) gradient of KCl in 0.02 M phosphate buffer and 3 0 ml fractions were collected. *b*, Enhanced mRNA release in response to the plasma fractions in *a* The data shown in the bar graph represent the net enhancement of mRNA release from prelabelled nuclei as % nuclear c.p m. by the addition to the minimal transport system of 0.3-ml aliquots of fractions 4, 8, 10, 11, or 12 of plasma from normal (hatched bars) or tumour-bearing (open bars) rats. The minimal transport system contained 4.5 × 10^{6} prelabelled liver nuclei per ml of medium containing 4 mg ml^{-1} of dialysed cytosol protein, 50 mM Tris HCl (pH 7 6), 25 mM KCl, 2 5 mM MgCl$_2$, 0 5 mM CaCl$_2$, 0 3 mM MnCl$_2$, 5 0 mM NaCl, 2 5 mM K$_2$HPO$_4$, 5 0 mM spermidine, 2.0 mM dithiothreitol, 3 0 mM ATP, 2.5 mM phosphoenol pyruvate, 35 units of pyruvate kinase and 300 mg of yeast RNA per ml, the total volume of the assay being 5.0 ml. The incubation was carried out at 30 °C for 30 min with the further addition of 1.0 mM phosphoenol pyruvate after 10 min and again after 20 min of incubation. To determine the percentage total of labelled nuclear RNA released during incubation, the RNA was precipitated from the nuclei-freed supernatants in the presence of carrier yeast RNA with 0.1 volume of 50% trichloroacetic acid, dissolved in solubiliser and counted in liquid scintillant[3]. During a 30-min incubation the release of total nuclear counts (± s.d.) for the minimal system was 4 7 ±0.2. Results are tabulated in terms of increment in % nuclear c.p.m. transported in the presence of the added plasma components over that released to the minimal system, and represent the average of two determinations which duplicated to within 10%. Essentially similar results were obtained whether or not the whole plasma was purified of low molecular weight components on Sephadex G-25. The experiments on normal serum and on hepatoma 5123D-bearing rats duplicated to within 10% They were also confirmed using hepatoma 9618A and the Novikoff hepatoma-bearing rats

hepatoma, with transplant generation times of 8 months and 1 week, respectively, were identical to that of the 5123D hepatoma. Also shown is the protein elution profile of the plasma from hepatoma 5123D, 18 d after inoculation, that is, when the tumour is not yet palpable. The protein elution profiles (Fig. 1) of the plasma from normal and hepatoma-bearing rats are markedly different. Thus, whereas the peak elution in the void volume (fractions 2–6) is common to the plasma of both the normal and tumour-bearing rats, the other two peaks are not. In fact, the protein components eluting at a mean KCl concentration of 0.05 M (fractions 7–10) disappears during the growth of the tumour, being decreased in the plasma from animals during the early stages of tumour growth and essentially absent during the later stages. In contrast, the protein components eluting at a mean KCl concentration of 0.125 M may be present at a very low level or essentially absent in normal rat plasma, but increase markedly during the development of the tumour. Identical results were obtained with animals bearing the Novikoff hepatoma, or hepatoma 9618A.

Shown in Fig. 1b is the ability of selected DEAE–cellulose fractions of plasma from a normal rat and a rat bearing hepatoma 5123D (tumour diameter of 1.0 cm) to enhance the transport of mRNA in the cell-free system. Fractions 4, 8 and 10 from both the normal and tumour-bearing animals showed comparable, but low transport activity. In contrast, fractions 11 and 12 had marked RNA transport activity when the plasma was derived from the tumour-bearing rat; the activity of the corresponding fractions from normal plasma was approximately one sixth that of plasma from the tumour-bearing rat. Again, the results obtained with rats bearing the Novikoff and 9618A hepatomas were identical to those reported for hepatoma 5123D. In contrast to the sixfold difference in transport activity observed when fractions 11 or 12 of the plasma from normal and tumour-bearing rats were compared, a difference of only threefold was observed when the crude plasmas from the two sources were compared.

The relationship, if any, between the disappearance during tumour growth, of the protein peak eluting from DEAE–cellulose at 0.05 M KCl and the appearance of the protein peak eluting at 0.125 M KCl, as well as the mechanisms underlying these changes are at present unknown. The possibility that the components eluting at 0.05 M KCl modify the transport activity of components eluting at 0.125 M KCl, however, was tested in the concentration range 1.0–3.0 mg ml^{-1}, with negative results.

The differential effect of active plasma fractions from normal and tumour-bearing rats on nuclear RNA restriction *in vitro* was tested by competitive RNA–DNA hybridisation. ^{14}C-labelled RNA transported from prelabelled liver nuclei in response to 4.0 mg ml^{-1} of homologous cytosol protein, fortified with 1.0 mg ml^{-1} of protein from fraction 12 (Fig. 1a) obtained by chromatographing the plasma of a Novikoff hepatoma-bearing rat, was competed against unlabelled RNA transported from unlabelled liver nuclei in response to homologous cytosol protein (4 0 mg ml^{-1}) plus fraction 12 (1.0 mg ml^{-1}), derived from normal plasma. Only 75 % of the messenger-like RNA sequences transported were identical in both systems, 25 % of the messenger-like RNA transported in the presence of the plasma protein fraction from normal animals being different from that transported in the presence of the comparable fraction from tumour-bearing rats. Furthermore, analysis of the transported labelled RNA in sucrose density gradients (results not shown) indicated that mRNA transported in the presence of plasma fractions of the hepatoma-bearing, in contrast to the normal rats, had a size distribution which was distinctly skewed to the heavier species; this abnormal size distribution was not normalised by active fractions from normal plasma.

Both the hybridisation and density gradient studies suggest that there are qualitative differences in the regulatory macro-molecules of the plasma from normal and tumour-bearing rats; it is not merely an increase in normal components. Since

analogous differences were observed[3] between the RNA transported to normal and tumour cytosols, some of the plasma components may originate from the tumour. It has been established[13,14] that certain RNA sequences are confined to the nucleus in normal cells but that this restriction is lost in cancer cells[15]. It has also been shown[11] that the enzyme composition of the host liver in animals bearing tumours shows a convergence to that of the corresponding tumour. Thus, our finding in the circulation of a regulatory component altering nuclear restriction is not surprising. Furthermore, there are several[16,17] recent reports of specific serum components disappearing as a tumour develops although as yet neither these components nor the factor eluting from DEAE–cellulose at 0.05 M KCl have been well characterised.

Although normal plasma also contains components which enhance RNA transport, they are present in low concentration compared with tumour plasma and elicit the transport of messenger-like RNA species which are different from those transported in the presence of plasma from tumour-bearing rats. Furthermore, the protein peak containing the highest transport activity in the plasma from tumour-bearing rats is essentially missing from normal plasma; it is also low (non-detectable) in the plasma of rats with regenerating liver (unpublished observations). These results strongly suggest, but do not prove that the increased transport activity in plasma from tumour-bearing animals is due to the release of macromolecules from tumour cells to circulation. The observation that the difference in transport ability of plasma from normal and tumour-bearing animals is enhanced by preliminary fractionation on DEAE–cellulose is relevant to the adaptation of the nuclear RNA restriction assay as a tumour detection test. But further correlative studies on animals bearing a wide range of transplantable, induced primary and autochthonous tumours of various sizes are required to establish the usefulness and limitations of this test system.

DOROTHY E. SCHUMM
THOMAS E. WEBB

Department of Physiological Chemistry,
The Ohio State University College of Medicine,
Columbus, Ohio 43210

1 Schumm, D. E , and Webb, T E., *J. natn. Cancer Inst.*, **54**, 123–128 (1975).
2 Schumm, D. E , and Webb, T E., *Biochem biophys. Res Commun.*, **48**, 1259–1265 (1972)
3 Schumm, D E , Morris, H. P., and Webb, T. E , *Cancer Res.*, **33**, 1821–1828 (1973).
4 Schumm, D. E., McNamara, D. J , and Webb, T. E., *Nature new Biol*, **245**, 201–203 (1973)
5 Schumm, D. E , and Webb, T E , *Biochem. J*, **139**, 191–196 (1974)
6 Yu, L C , Racevskis, J., and Webb, T. E , *Cancer Res.*, **32**, 2314–2321 (1971).
7 Racevskis, J., and Webb, T. E , *Eur. J. Biochem.*, **49**, 93–100 (1974).
8 Schumm, D E , and Webb, T. E., *Biochem. biophys. Res. Commun*, **58**, 354–360 (1974)
9 Bullough, W S , and Lawrence, E. B , *Nature*, **220**, 137–138 (1968)
10 Rytoma, T., and Kiviniemk, K., *Nature*, **222**, 995–996 (1969)
11 Herzfeld, A , and Greengard, O., *Cancer Res.*, **32**, 1826–1832 (1972).
12 Morris, H P , and Wagner, B. P., in *Methods in Cancer Research*, **4** (edit. by Busch, H), 125–137 (Academic, New York, 1968).
13 Drews, J , Brawerman, G., and Morris, H P., *Eur. J Biochem* , **3**, 284–292 (1968).
14 Birnie, B D., *Proc XIth Int. Cancer Congress*, Florence (in the press)
15 Sporn, M B., *Biochem. Pharm* , **20**, 1029 (1971)
16 Dolan, M L , Coetzee, M. L., Spangler, M., and Ove, P , *Cancer Res*, **34**, 3010–3017 (1974).
17 Ove, P , Coetzee, M L, Obenrader, M., and Short, T., *Oncology*, **29**, 13–21 (1974)

Early role during chemical evolution for cytochrome P450 in oxygen detoxification

IT is generally held that early in the evolutionary history of the Earth, when abiogenic chemicals were forming associations possessing attributes by which we now define living organisms, this planet had a reducing atmosphere. The

subsequent appearance of free or molecular oxygen must have had a profound effect on such poorly specialised life forms and many would have perished. It was long assumed that the adaptive enzymatic changes of the successful forms were chiefly the elaboration of the respiratory chain enzymes, peroxidases and catalase. Research on superoxide dismutase[1] has suggested that this enzyme may also have controlled oxygen toxicity in ancient living tissue. We have studied cytochrome P450, which also metabolises oxygen, and believe that this protein may possibly be more primitive than those considered previously and may even have emerged before the advent of atmospheric oxygen.

Cytochrome P450 is an unusual haemprotein found in animals, plants and microorganisms. In animals, this b-type cytochrome is membrane-bound and is present in the mitochondria of certain endocrine glands and in the microsomes of cells of, among others, certain endocrine glands and the liver. Microsomal cytochrome P450 is the major hepatic haemprotein, reaching concentrations of 0.1 mM in the cell or five to ten times that of any mitochondrial (respiratory chain) cytochromes[2]. In certain conditions, cytochrome P450 constitutes 20% of the microsomal protein or 2–3% of the hepatic protein content[2]. The *Pseudomonas* enzyme, in contrast, is a soluble protein[3,4].

The cytochromes P450 possess active sites effecting hydroxylation reactions

$$R-H + O_2 + 2e = R-OH + H_2O$$
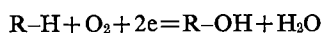
The two electrons or reducing equivalents are furnished by NADPH and (or) NADH (ref. 5). The first reduction is of a flavoprotein but iron–sulphur proteins also participate in electron transfer for mitochondrial and bacterial hydroxylases[5,6]. The biological functions of cytochrome P450-dependent hydroxylases include the hydroxylations (often detoxifications) of many structurally dissimilar substances such as steroids and fatty acids and xenobiotics such as drugs, carcinogens, pesticides, cannabinoids and a variety of substituted and unsubstituted hydrocarbons[5,7-10]. Cytochrome P450 may also act as a microsomal peroxidase[11].

Both iron–sulphur proteins and cytochrome P450 possess low redox potentials[6,12,13], close to that of the hydrogen electrode (−420 mV), unlike other cytochromes and other terminal oxidases. They could thus have functioned enzymatically at a time when the Earth's atmosphere was mainly composed of hydrogen, nitrogen, methane, ammonia, carbon monoxide and carbon dioxide[14,15], and also after free oxygen had appeared. Both are relatively small, single polypeptides (iron–sulphur proteins, molecular weight 6,000–12,000; bacterial cytochrome P450, 45,000) with low isoelectric points; have a large content of amino acids which can be synthesised abiologically[14]; and can be reconstituted from their apoproteins *in vitro*[4,16,17]. Both types are widely distributed in microorganisms, plants and animals[5,6,18] and participate in the metabolism of a large range of substrates[5,6,9]. The above characteristics have led some to consider that the iron–sulphur proteins may have been among the earliest to evolve and that this process took place in reducing conditions[6].

The gradual appearance of molecular oxygen would have led to the development in primitive organisms of biochemical mechanisms either as lines of defence (for example, utilisation of porphyrins[19]) or to benefit from the alteration in environment (for example, development of the energy-yielding respiratory process). Although molecular oxygen is itself damaging to living matter (for example, enzymes which are labile to aerobic conditions[17], autoxidation of lipids and oxidation of free sulphydryl groups of proteins), products of oxygen metabolism such as superoxide anion, H_2O_2 and OH^0 are also highly reactive and toxic to the living fabric It seems unlikely that the toxicity of

oxygen[19,20] is caused by a single toxic action, and the development of tolerance is probably multifactorial[20]. The rate of production of the superoxide radical by some enzymes has also been shown to depend on oxygen tension[20]. The early organism may thus have developed different lines of defence against damage by the steadily increasing atmospheric oxygen. Initially, cytochrome P450 alone may have been adequate to 'mop up' traces of unwanted oxygen which entered the cell. Later, cytochrome P450 activity may not have been adequate by itself to remove all the excess oxygen but would have still performed a valuable 'buffering' or attenuating role in lowering the production of H_2O_2 and superoxide anion to within limits which could be effectively detoxified by other enzymes. This activity may also have provided the time necessary for the induction of enzymes such as superoxide dismutase[1] whenever the organism was challenged by elevated oxygen levels in the immediate environment. Occasionally, enzyme systems may produce under certain chemical constraints some reactive metabolites of oxygen such as H_2O_2 and superoxide anion. These would have been balanced by the concerted action of enzymes such as cytochrome P450, superoxide dismutase and catalase, thus reducing the longevity of these toxic species in the cellular substance. Catalase is possibly of lesser importance in protecting bacteria against oxygen toxicity[1] and the innocuous nature of the products of the activity of cytochrome P450 may be of advantage. The superoxide anion and peroxide which may be produced in certain conditions *in vivo* by cytochrome P450-dependent enzyme systems (but see ref. 11), however, would at that time, as now, have required rapid detoxification by superoxide dismutase, peroxidase and catalase.

The apoprotein of cytochrome P450 could have been derived from abiogenic amino acids, and Calvin[16] has discussed how certain haemproteins may have been formed abiologically. The system would have been insensitive[7,8] to the levels of cyanide present in the primaeval oceans[14,15]. Chemical (or photochemical[6]?) reduction of an associated iron–sulphur protein suffices for the supply of electrons to cytochrome P450 in the absence of flavoproteins and reduced cofactors[6]. Although such reduction of an associated protein may have produced electrons for early cytochrome P450-dependent activities, Oparin[21] considers that cofactors must have appeared very early in chemical evolution. It is also relevant that the haemprotein complexes with phospholipids to form a proteolipid which is soluble in hydrocarbon solvent and may be functional in lipid surroundings[22]. This could be pertinent to the theories that primordial cells were highly lipid in nature[15].

The carbon monoxide in the atmosphere and the absence of oxygen[14,15] would have made unlikely the activity of cytochrome P450 as a hydroxylase very early in the evolution of life although the photochemical action spectrum of these hydroxylation reactions[5] has shown that illumination at certain wavelengths does reverse the inhibition caused by carbon monoxide. The solar energy which traversed the primitive atmosphere to reach the Earth's surface was very large[14,15]—sufficient to dissociate any carbon monoxide bound on to early forms of cytochrome P450. Cytochromes are proposed to have been among the active proteins which had evolved before the advent of the oxygen atmosphere[23,24], and, in spite of the aerobic conditions in the liver cell, a subsidiary function of the contemporary hepatic microsomal enzyme may be in reductive reactions[10]. The low redox potentials of this cytochrome and associated proteins would have enabled similar reductive activities of early cytochrome P450 to have taken place in an anaerobic hydrogen atmosphere. Contemporary cytochrome P450 may also have a role in nitrogen reduction and appears in *Rhizobium* sp. bacteroids by the time of active nitrogen fixation, which requires both nitrogen and oxygen[3,25]. Although research on cytochrome P450 has focused mainly

on its activities in an aerobic environment, it would be significant to demonstrate that this haemprotein exists in contemporary bacteria living in anaerobic conditions.

Pseudomonas and mitochondrial cytochromes P450 differ from microsomal cytochrome P450 in requiring the functioning of iron–sulphur proteins[5]. Both the mitochondrial cytochrome P450 and the associated iron–sulphur protein are located on the inner mitochondrial membrane[17]. Mitochondria may have been symbiotic inclusions in eukaryote development[26], and this fundamental difference between mitochondrial and microsomal cytochromes P450 would further suggest that cytochrome P450-dependent enzyme systems were evolutionary entities which had developed along separate lines in different prokaryotes before the formation of the eukaryotic cell. Possibly relevant here is the hypothesis[27] that the ancestors of the prokaryotes were simple abyssal organisms The ancestors of the eukaryotes, however, developed near the surface of the waters where they were exposed to light and, in due course, to the oxygen atmosphere. The exposure to light of the surface organisms would have reversed any inhibition of cytochrome P450 by atmospheric carbon monoxide[5] in the early period, and the proximity to atmospheric oxygen at a later stage may have favoured surface organisms possessing cytochrome P450. Note also that this enzyme has been found in a variety of contemporary higher organisms but few microorganisms[5,18].

If the hypothesis presented here is correct, a form of cytochrome P450 has been present in life forms for billions of years[15,26] and probably before the advent of free oxygen and the development of other respiratory haemproteins. When its protective value in detoxifying oxygen was superceded by subsequent development of aerobic organisms[26], the hydroxylase was retained because of its usefulness in biosynthetic processes and in the degradation of varied complex molecules.

We thank Dr J. Canick for comments and discussion. This work was supported by the National Cancer Institute, NIH.

Rohan H. Wickramasinghe
Claude A. Villee

*Laboratory of Human Reproduction
and Reproductive Biology,
Department of Biological Chemistry,
Harvard Medical School, 25 Shattuck Street,
Boston, Massachusetts 02115*

1 Fridovich, I , *Adv Enzym* , 41, 35–97 (1974)
2 Estabrook, R W , Franklin, M , Baron, J., Shigematsu, A., and Hildebrandt, A , in *Drugs and Cell Regulation* (edit. by Mihich, E.), 227 (Academic, New York, 1971)
3 Tsai, R L., Gunsalus, I C , and Dus, K , *Biochem. biophys Res. Commun* , 45, 1300–1306 (1971)
4 Yu, C.-A , Ganguli, B N , Bartholomaus, R. C., and Gunsalus, I C , *Bact. Proc* , 119 (1969)
5 Wickramasinghe, R. H., *Enzyme*, 19, 348–376 (1975).
6 Wickramasinghe, R H , and McIntosh, E. N , *Enzyme*, 17, 210–226 (1974), Wickramasinghe, R H., *Enzyme*, 17, 227–264 (1974)
7 Cammer, W , and Estabrook, R W , *Archs Biochem. Biophys* , 122, 721–734 (1967).
8 Cardini, G , and Jurtshuk, P , *J. biol Chem.*, 245, 2789–2796 (1970)
9 Gillette, J R , *et al* , (eds), *Microsomes and Drug Oxidations* (Academic, New York, 1969)
10 Gillette, J R , Davis, D. C., and Sasame, H. A , *A Rev. Pharmac.*, 12, 57–84 (1972)
11 Hrycay, E G., and O'Brien, P J , *Archs Biochem. Biophys.*, 153, 480–494 (1972). Rahimtula, A D., and O'Brien, P J , *Biochem biophys Res Commun.*, 60, 440–447 (1974).
12 Schleyer, H , Cooper, D. Y , and Rosenthal, O., *Fedn Proc* , 29, A925 (1970)
13 Waterman, M R., and Mason, H S., *Biochem biophys Res. Commun.*, 39, 450–454 (1970)
14 Ponnamperuma, C., (ed), *Exobiology* (North-Holland, Amsterdam, 1972)
15 Ponnamperuma, C., *The Origins of Life* (Thames and Hudson, London, 1972)
16 Calvin, M , *Am Scient* , 44, 248–263 (1956).
17 Wickramasinghe, R H., *Space Life Sci.*, 4, 341–352 (1973)
18 Potts, J R. M , Weklych, R , and Conn, E E , *J biol Chem.*, 249, 5019–5026 (1974)
19 Ponnamperuma, C , and Gabel, N W , *Space Life Sci* , 1, 64–96 (1968)
20 Crapo, J D , and Tierney, D F , *Am J. Physiol* , 226, 1401–1407 (1974).
21 Oparin, A I , in *Exobiology* (edit by Ponnamperuma, C), 1 (North-Holland, Amsterdam, 1972)
22 Wickramasinghe, R. H , *Cytobios*, 8, 81–94 (1973)
23 Lascelles, J , in *Oxygen in the Animal Organism*, IUB Symp. series, 31, 657 (Macmillan, London, 1964)
24 Mason, H. S , in *Homologous Enzymes and Biochemical Evolution* (edit by Thoai, N. V , and Roche, J), 69 (Gordon and Breach, New York, 1968)
25 Matus, V. K , Melik-Sarkisian, S S , and Kretovick, V. L., *Mikrobiologiia*, 42, 112–118 (1973). Appleby, C A., *Biochim biophys Acta*, 188, 222–229 (1969)
26 Margulis, L , *Origin of Eukaryotic Cells* (Yale University Press, New Haven, 1970)
27 Sagan, C., *J. theoi. Biol.*, 39, 195–200 (1973)

Human embryonic haemoglobins including a comparison by homology of the human ζ and α chains

DURING the first 10 weeks of human embryonic life, there exist unique haemoglobins designated Gower 1 and Gower 2, and it has been suggested that Hb Gower 1 is a tetramer consisting of ε chains (ε₄) (ref. 1). Haemoglobin Portland has also been described in infants with developmental abnormalities[2] and neonates with severe α–thalassaemia, the Bart's hydrops foetalis syndrome[3,4]. Hb Portland is composed of two γ chains and two ζ chains[5].

The data of Capp, Rigas and Jones[5] on the ζ chain tryptic peptides suggested an α chain-like structure because of the presence of the dipeptide Tyr–Arg, which is unique for the C-terminal part of the α chain, rather than Tyr–His which is unique for human non-α chains. A more specific unique property of the α chain is its lack of the D helix (residues 50–56), and when in a second analysis of the ζ chain the alignment of the peptides was incompatible with the presence of the D helix, this strongly suggested that the ζ chain was indeed an early α chain[6]. Haemoglobin function requires two α and two non-α chains[7] and because embryonic and foetal cells showed similar

Fig. 1 Maps of the soluble tryptic peptides (a) and the peptic peptides (b) of the tryptic "core" from 8 mg of globin from Hb Portland, prepared as described previously[9], electrophoresis at pH 6.4 for a and pH 3.5 for b. Tryptophan-containing peptides were located under an ultraviolet lamp, and the positions of the remaining peptides were determined using fluorescamine. The ζ-chain peptides are shaded. Peptides A (Ala₂, Phe, Arg) and B (Ala₂ Arg) could not be aligned with any of the known α-chain sequences and are suggestive of a sequence Phe–Ala–Ala–Arg, Peptide Y described earlier[5,6] is suggestive of the α-chain elongation in Hb Icaria[8,18]

```
                                10                                                    20
α  Val------Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Val-Lys-Ala-Ala-Trp-Gly-Lys-Val-Gly-Ala-His-Ala-Gly-Glu-Tyr-Gly-Ala-Glu-Ala-Leu-
ζ  (Gly    Leu-Ser Ile Ala Asp Lys)(Thr Ser Leu Lys)(Asn Ala Trp Gly Lys)(Ile Ser Thr Asp Thr Thr Glu Ile Gly Thr Glu Ala Leu
                                10                                                    20
β  Val-His-Leu-Thr-Pro-Glu-Glu-Lys-Ser-Ala-Val-Thr-Ala-Leu-Trp-Gly-Lys-Val-Asn------Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-
γ  Gly-His-Phe-Thr-Glu-Gly-Asp-Lys-Ala-Thr-Ile-Thr-Ser-Leu-Trp-Gly-Lys-Val-Asn      Val-Glu-Asp-Ala-Gly-Gly-Glu-Thr-Leu-
Helix    NA    ►◄        A                                          ►◄                              B
                                                                                          50
         30                                                                           50
       o  o  Δ    o  o  o  ●  ●  Δ  ●  ●  ●     Δ  Δ  ●      Δ  Δ
α  Glu-Arg-Met-Phe-Leu-Ser-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe------Asp-Leu-Ser-His-----------------Gly-Ser-Ala-
ζ  Glu Arg)(Leu His Leu Ser Phe Pro Thr Gln Lys)                              (Phe Leu Ser His               Gly Leu Ala
         30                                                40                          50
β  Gly-Arg-Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-Phe-Glu-Ser-Phe-Gly-Asp-Leu-Ser-Thr-Pro-Asp-Ala-Val-Met-Gly-Asn-Pro-
γ  Gly-Arg-Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-Phe-Asp-Ser-Phe-Gly-Asn-Leu-Ser-Ser-Ala-Ser-Ala-Ile-Met-Gly-Asn-Pro-
Helix       B                   ►◄   C                ►◄    CD       ►◄       D               ►◄    E
                              60                              70                    80
                      Δ      Δ                Δ  ●     Δ  ●  ●  ●     Δ  Δ           Δ       o  o      o  o           o
α  Gln-Val-Lys-Gly-His-Gly-Lys-Lys-Val-Ala-Asp-Ala-Leu-Thr-Asn-Ala-Val-Ala-His-Val-Asp-Asp-Met-Pro-Asn-Ala-Leu-Ser-Ala-Leu-
ζ  His Val Lys)(Ala His Gly Ser Lys)(Val Ala Gly Ala Leu Thr Ser Ile Leu Gly Pro Val Asp Ser Phe Lys)(Asn Ala Val Gly Ala Leu
         60                              70                              80
β  Lys-Val-Lys-Ala-His-Gly-Lys-Lys-Val-Leu-Gly-Ala-Phe-Ser-Asp-Gly-Leu-Ala-His-Leu-Asp-Asn-Leu-Lys-Gly-Thr-Phe-Ala-Thr-Leu-
γ  Lys-Val-Lys-Ala-His-Gly-Lys-Lys-Val-Leu-Thr-Ser-Leu-Gly-Asp-Ala-Ile-Lys-His-Leu-Asp-Asp-Leu-Lys-Gly-Thr-Phe-Ala-Gln-Leu-
Helix            E                ►◄              E                        ►◄    EF          ►◄    E
                       90                              100                          110
α  Ser-Asp-Leu-His-Ala-His-Lys-Leu-Arg-Val-Asp-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ser-His-Cys-Leu-Leu-Val-Thr-Leu-Ala-Ala-His-Leu-
ζ  Ser Glu Val His Ala Lys)(Ile Leu Arg)(Val Asp Pro Val Asn Phe Arg)(Ala Leu Ser His Cys Leu Leu Gly Lys)(Ser Leu Glx Ala His Leu-
                       100                              110
β  Ser-Glu-Leu-His-Cys-Asp-Lys-Leu-His-Val-Asp-Pro-Glu-Asn-Phe-Arg-Leu-Leu-Gly-Asn-Val-Leu-Val-Cys-Val-Leu-Ala-His-His-Phe-
γ  Ser-Glu-Leu-His-Cys-Asp-Lys-Leu-His-Val-Asp-Pro-Glu-Asn-Phe-Lys-Leu-Leu-Gly-Asn-Val-Leu-Val-Thr-Val-Leu-Ala-Ile-His-Phe
Helix    F              ►◄        FG           ►◄              G               ►◄   130                                 ►◄   GH
                                   120                                       130                              140
α  Pro-Ala-Glu-Phe-Thr-Pro-Ala-Val-His-Ala-Ser-Leu-Asp-Lys-Phe-Leu-Ala-Ser-Val-Ser-Thr-Val-Leu-Thr-Ser-Lys-Tyr-Arg
ζ  Tyr Ala Glx Phe Thr Ile Gly Val Asx Ala Ser Leu Asp Lys)(Phe Leu Ala Ser Val Ser Thr Val Leu Glx Ser Lys)(Tyr Arg)
         120                                    130                               140
β  Gly-Lys-Glu-Phe-Thr-Pro-Pro-Val-Gln-Ala-Ala-Tyr-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys-Tyr-His
γ  Gly-Lys-Glu-Phe-Thr-Pro-Glu-Val-Gln-Ala-Ser-Trp-Gln-Lys-Met-Val-Thr-Gly-Val-Ala-Ser-Ala-Leu-Ser-Ser-Arg-Tyr-His
Helix       GH              ►◄                          H                        ►◄                      ►◄    HC
```

Fig. 2 Alignment of ζ-chain peptides with the amino acid sequences of three human haemoglobin chains. The chains have been arranged so as to maximise homologies, also taking into account the polarity and size of the residues, the haem contacts and the intramolecular contacts of α and β chains according to the model of Perutz as applied to man[19,20]. The alignment suggests that the ζ chain is very closely related to the α chain. O, α1β1 contacts; ●, α1β2 contacts; Δ, haem contacts.

oxygen dissociation curves, Huehns and Farooqui[8] concluded that the ζ chain ought to resemble the α chain.

We have examined human embryos aged 5–10 weeks and neonates with the Hb Bart's hydrops foetalis syndrome for Hb Gower 1 and Portland. The Hb Gower 1 fraction contains α chains and therefore Hb Gower 1 is unlikely to be a tetramer of ε chains. In the case of Hb Portland we have been able to obtain further evidence for resemblance of the ζ chain to the α–chain.

We obtained blood from 67 embryos. The red cells were washed several times in excess physiological saline and haemolysed as previously described[9]. The haemolysates were pooled and chromatographed on DEAE–Sephadex[10] and the fractions eluting earlier than Hb A₂, were pooled, concentrated by ultrafiltration, then electrophoresed on paper at pH 8.9 to isolate the embryonic haemoglobins. Fractions corresponding to Hb Gower 1 and Gower 2 were eluted with water and pooled separately and concentrated by ultrafiltration at 40 °C.

When the Gower 1 fraction was chain separated on a CM cellulose column[11] the major peak which was obtained gave on subsequent fingerprinting and amino acid analysis, peptides with amino acid compositions consistent with the structure of carbonic anhydrase B[12]. A tryptic dipeptide Gly-Arg which has been cited as an example of an ε-chain peptide[13], is also found in carbonic anhydrase B. The peak in position of the α chain was a haemoglobin α chain from fingerprinting and amino acid analysis of all soluble peptides.

To investigate the ζ chain, blood was obtained from two Hb Bart's hydrops foetalis neonates and Hb Portland was isolated by paper electrophoresis; haem was then removed and the globin dried under nitrogen[9]; 8 mg were digested with trypsin

and pepsin and fingerprints prepared (Fig. 1); 1 mg was used for the terminal amino acid sequence, using the modified dansyl technique of Gray[14,15]. Glycine only was released. Most of the peptides reported by Capp et al.[5] were obtained and the alignment of peptides is similar to that reported previously[6]. We have been able to align 14 additional residues and the only part of the α chain for which no peptide suitable for alignment was found are residues 41–46. We have, however, interchanged Phe and Leu for positions 47 and 52, because of the alignment of a dipeptide Gly–Leu to positions 51–52 (Fig. 1).

It is suggested from the electrophoretic mobility of peptide X aligned with residues 1–7, that the N terminus may be blocked, possibly through acetylation as it is in some γ chains. The corresponding non-blocked peptide with the expected electrophoretic mobility was not found. Of the 15 haem contacts available for comparison, (that is residues 32, 39, 58, 62, 83, 86, 87, 91, 93, 97, 98, 101, 129, 132 and 136) 12 can be aligned to be identical to those of α chain. Of the 30 internal residues available, 22 are alike, 7 very similar and only one dissimilar to those of α chains (α32). Of the subunit contacts, the 16 α1β1 contact alignments accommodate identical residues at 11 positions; the five others are 106 Leu/Gly, 107 Val/Lys, 114 Pro/Tyr, 119 Pro/Ile and 122 His/Asx. All the 10 α1β2 contacts available for comparison are identical to those of α chains.

To determine whether substantial amounts of Hb Portland occur in normal embryos, three additional embryos below the age of 10 weeks were later obtained and from the haemolysates, separated globins were prepared, digested with trypsin and fingerprints were prepared as above. No ζ chain peptides were detected. If Hb Portland occurs in normal embryos, it must occur at a concentration well below 10%. Purified Hb Portland

is known to have a cooperative oxygen dissociation curve with a reduced alkaline Bohr effect[16]. If it formed 40% of the haemoglobin in embryonic cells[17] it would be expected to affect their Bohr effect and cause them to differ from foetal cells rather than resemble them, as has been reported[4]. Hb Gower 1, if it is an ε_4 tetramer, should raise the oxygen affinity, as tetramers of like chains have very high oxygen affinities[7]. We conclude that if erythrocytes of young human embryos, aged 5–10 weeks resemble foetal cells in oxygen affinity and Bohr effect[8] they are unlikely to contain substantial amounts of Hb Portland and Hb ε_4.

H. KAMUZORA*
H. LEHMANN

MRC Abnormal Haemoglobin Unit,
University Department of Clinical Biochemistry,
Addenbrooke's Hospital,
Hills Road, Cambridge CB2 2QR, UK

*H K is a Commonwealth Scholar, Churchill College, Cambridge, on study leave from the Faculty of Medicine, University of Dar-Es-Salaam, Tanzania

1 Huehns, E. R , Dance, N., Beaven, G. H , Hecht, P , and Motulsky, A. G , Cold Spring Harb Symp quant. Biol , 29, 327–331 (1964)
2 Capp, G. L., Rigas, D A , and Jones, R T , Science, 157, 65–66 (1967)
3 Weatherall, D J., Clegg, J B., and Boon, W H., Br J Haemat., 18, 357–367 (1970).
4 Todd, D., Lai, M C S , Beaven, G H., and Huehns, E R., Br. J Haemat., 19, 27–31 (1970)
5 Capp, G L., Rigas, D A , and Jones, R T , Nature, 228, 278–280 (1970)
6 Kamuzora, H , Jones, R. T , and Lehmann, H , FEBS Lett., 46, 195–199 (1974)
7 Perutz, M F , and Mazarella, H , Nature, 199, 633–639 (1963)
8 Huehns, E. R , and Farooqui, A M., Nature, 254, 335–337 (1975)
9 Lehmann, H , and Huntsman, R G , Mans' Haemoglobins, second ed. (North Holland, Amsterdam, 1974)
10 Huisman, T H J , and Dozy, A M , J Chrom 19, 160–169 (1965).
11 Clegg, J B , Naughton, M. A., and Weatherall, D J , J molec Biol 19, 91–108 (1966).
12 Kuang-Tzu, D. L N , and Deutsch, H F , J biol Chem 248, 1885–1893 (1973),
13 Szelenyi, J F , and Hollan S. R , Acta biochim biophys Acad Sci Hungary, 4, 47–55 (1969)
14 Gray, W. R., Methods in Enzymology, (edit by Colowick, S P , and Kaplan, N. O), 11, 139–150 (Academic, New York and London, 1967)
15 Weiner, A M., Platt, T , and Weber, K , J biol Chem 247, 3242–3251 (1972)
16 Tuchinda, S., Nagai, K , and Lehmann, H., FEBS Lett , 49, 390–391 (1975)
17 Hecht, F , Motulsky, A. G., Lemire, R F., and Shepard, T D , Science, 152, 91–92 (1966)
18 Clegg, J. B , et al , Nature, 251, 245–247 (1974)
19 Perutz, M F , Nature, 228, 726–739 (1970)
20 Perutz, M F., and Ten Eyck, L. F , Cold Spring Harb Symp. quant Biol , 36, 295–310 (1971)

Lycorine as an inhibitor of ascorbic acid biosynthesis

LYCORINE, an alkaloid extracted from Amaryllidaceae[1,2], is a powerful inhibitor of growth in higher plants, algae and yeasts. At the very low concentration of 10^{-6} M, lycorine inhibits cell division in higher plants, red algae and yeasts, and the cell cycle is arrested in the interphase stage[3]. No inhibition of cell division was observed in Escherichia coli (O. A., unpublished).

Lycorine also inhibits expansion growth in higher plants[3].

At 10^{-4} M, while strongly inhibiting expansion growth, lycorine induces the following metabolic responses[4]: inhibition of ^{14}C-leucine incorporation into proteins; subsequent significant inhibition of ^{3}H-uridine incorporation into RNA; and a massive drop in the ascorbic acid (AA) content and a corresponding increase in dehydroascorbic acid (DHA). The effect of lycorine on protein synthesis was clearly an indirect one, as isolated polysomes were found to be lycorine-insensitive. Because RNA synthesis is only inhibited as a long term effect, it was suggested that the primary inhibiting effect of lycorine could be its action on the ascorbate system[3,4].

To demonstrate lycorine–ascorbic acid interaction, attempts were made to remove the effect of lycorine by exogenous administration of AA. This kind of experiment was not successful in higher plants, however, because AA was promptly oxidised by the tissue, mainly at the cut surfaces, and DHA accumulated in the cell, thus inhibiting plant growth. In contrast, the inhibition of growth induced by lycorine in red algae was almost completely overcome by the addition of external AA[5]. This was obtained because AA is very slowly oxidised to DHA on the cut surfaces so that there is no DHA accumulation in the cell, whereas AA does accumulate and thus overcomes the lycorine effect. These results show that lycorine does not interfere with AA utilisation in red algae and strongly suggest that the effect of lycorine on plants could be the result of its ability to inhibit AA biosynthesis.

To determine the effect of lycorine on AA biosynthesis, 3-d-old seedlings of Pisum sativum, 2 cm long and deprived of their roots, were kept in distilled water (or in lycorine, where indicated) in the dark for 18 h (starvation) and were subsequently exposed to light for 14 h. AA content of seedlings diminished during starvation: the total amount of AA+DHA decreased from 6,763 γ per g dry weight to 3,672 γ per g dry weight (Table 1). This means that a large amount of AA is utilised in cell metabolism[6]. Cells corresponding to 1 g dry weight utilise about 172 γ h^{-1} AA in their metabolism and growth.

Actually, the amount of AA utilised in cell metabolism is higher; in fact the decrease in AA content is stronger in the presence of lycorine. With 6×10^{-5} M lycorine present during starvation, the total amount of AA is only 1,305 γ per g dry weight (Table 1). These data seem to suggest that the seedlings synthesise a certain amount of AA during starvation in the dark, as they utilise the reserve carbohydrates that are present in parenchymatous cells; this does not occur with lycorine and the AA content is consequently lowered at the end of starvation. AA biosynthesis inhibition by lycorine is better evaluated when the seedlings are illuminated after the starvation period (Table 2) In these conditions the controls synthesise a considerable amount of AA: total content (AA+DHA) rises from 3,646 to 4,776 γ per g dry weight. Lycorine present in the medium during light exposure strongly inhibits AA biosynthesis, the rate of inhibition being 41% at 5×10^{-6} M and 90% at 2×10^{-5} M. Considering that lycorine enters the seedlings only through the lower cut surface of the stem, it is very likely that lycorine concentration is quite low in the leaves where large amounts of AA are synthesised.

	Table 1 Effect of lycorine on AA content in rootless pea seedlings during starvation				
Time	Treatment	AA	DHA	Total	AA + DHA utilised
0		5,411	1,352	(γ per g dry weight) 6,763	in metabolism —
Starvation	Water	2,800	872	3,672	3,091
Starvation	Lycorine 10^{-5} M	2,300	640	2,940	3,823
Starvation	Lycorine 3×10^{-5} M	1,910	535	2,445	4,318
Starvation	Lycorine 6×10^{-5} M	905	400	1,305	5,458

Seeds of Pisum sativum var Alaska, soaked 18 h in tap water, were planted in vermiculite wetted with water, and then placed in a dark environmental chamber at 27 °C; 3-d-old seedlings, 2 cm long, were deprived of their roots and starved in the dark, at 27 °C for 18 h The seedlings were arranged on Petri dishes through the perforated cover (25 for each dish). AA and DHA were assayed on the apices of seedlings; 50 apices for each sample, about 250 mg fresh weight, were homogenised in 8 ml 5% metaphosphoric acid and centrifuged. The supernatant was used for AA and DHA assay using 2,4-dinitrophenylhydrazine reaction[5,10,11]. All values reported are average determinations on triplicate samples.

Table 2 Effect of lycorine on AA synthesis in pea seedlings

Time	Treatment	AA	DHA	Total (γ per g dry weight)	AA + DHA synthesised	Inhibition (%)
0		5,338	1,332	6,670	—	—
Starvation		2,782	864	3,646	—	—
14 h exposure to light	*Water	3,679	1,097	4,776	1,130	—
14 h exposure to light	*Lycorine 5 × 10⁻⁶ M	3,334	978	4,312	666	41
14 h exposure to light	*Lycorine 10⁻⁵ M	3,250	893	4,143	497	56
14 h exposure to light	*Lycorine 2 × 10⁻⁵ M	3,031	728	3,759	113	90

* After 18 h starvation in water the seedlings were transferred on to other Petri dishes containing either water or lycorine and placed in an aerated chamber at 1,000 lx and 27 °C for 14 h.

Table 3 Effect of lycorine on AA synthesis in potato slices

	Treatment	AA	DHA	Total (γ per g dry weight)	AA + DHA synthesised	Inhibition (%)
Fresh slices		585 8	30.2	616	—	—
Activated slices	Water	839	47	886	270	—
Activated slices	Lycorine 5 × 10⁻⁷ M	760	50	810	194	28
Activated slices	Lycorine 10⁻⁶ M	704	49	753	137	50
Activated slices	Lycorine 3 × 10⁻⁶ M	600	45	645	29	89

Tuber slices of *Solanum tuberosum* L. were prepared as follows: cylinders of potato tissue (0.9 cm diameter) were removed with a cork borer and sliced on a sliding microtome. Slices (1 mm thick) were washed repeatedly in tap water and these were indicated as 'fresh' slices, whereas 'activated' slices[12] were those placed either in water or in lycorine for 24 h with air bubbled into the medium. AA and DHA determinations were made on 20 slices, about 2,500 mg fresh weight for each sample, and homogenised in 12 ml 5% metaphosphoric acid. All values reported are average determinations run on triplicate samples

Table 4 Effect of lycorine on AA synthesis in *Clivia* leaves

Time	Treatment	AA	DHA	Total (γ per g fresh weight)	AA + DHA synthesised	Inhibition (%)
0		1,584	104	1,688	—	—
Starvation		915	85	1,000	—	—
14 h exposure to light	Water	1,395	92	1,487	487	—
14 h exposure to light	Lycorine 10⁻⁵ M	1,410	90	1,500	500	—
14 h exposure to light	Lycorine 5 × 10⁻⁵ M	1,312	86	1,398	398	18

Young leaves of *Clivia miniata* Reg. were kept for 18 h in dark environmental chamber at 27 °C (starvation) The leaves were then placed in aerated chamber at 1,000 lx and 27 °C for 14 h. AA and DHA were assayed as indicated in Table 1, by homogenising 1 g fresh weight in 16 ml 5% metaphosphoric acid.

A demonstration of the extreme sensitivity of the AA biosynthesis system to lycorine has been provided using potato slices[7]. In contrast to pea seedlings, there are no permeability problems. In 'activated' slices, 5 × 10⁻⁷ M lycorine already inhibits 28% AA biosynthesis; almost complete inhibition is obtained at 3 × 10⁻⁶ M lycorine (Table 3).

Considering that AA biosynthesis is highly sensitive to lycorine (3 μM lycorine inhibits 89%); that lycorine does not interfere directly with protein and nucleic acid synthesis[4]; it does not affect either permeability or phosphorylation in mitochondria and chloroplast (O. A., unpublished); it does not affect animals that are not capable of synthesising AA but does induce scurvy-like symptoms in AA-synthesising animals[8,9], it seems possible to conclude that lycorine is quite a specific inhibitor of AA biosynthesis in both plants and animals, and that its biological effects are probably the result of this peculiar action.

Finally, within the Amarillidaceae, results show that AA biosynthesis is much less affected by lycorine in *Clivia miniata*—an Amarillidacea containing lycorine in both leaves and rhizomes[13]—than it is in pea and potato. Lycorine (10⁻⁵ M), although inhibiting 56% in pea seedlings and 100% in potato slices, does not affect AA biosynthesis in *Clivia* leaves: at lycorine concentrations five times higher, the inhibition is only 18% (Table 4).

These data raise an interesting question, particularly in view

of the phylogenetic implications—what is the relationship between the AA biosynthetic pathway and the presence of lycorine in Amarillidaceae? Before any conclusions can be drawn, however, our data should first be confirmed using the isolated AA biosynthetic system.

ORESTE ARRIGONI
ROSALIA ARRIGONI LISO
GIUSEPPE CALABRESE

Institute of Botany,
University of Bari,
70126 Bari, Italy

1 Nakagawa, Y , Ugeo, S , and Vajima, H., *Chemy. Ind*, **42**, 1238–1243 (1956)
2 Amico, A , Bruno, S , and Papa, F , *Flora salutaris*, **5**, 1–7 (1967).
3 De Leo, P , Dalessandro, G , De Santis, A., and Arrigoni, O., *Pl. Cell Physiol*, **14**, 481–486 (1973)
4 De Leo, P , Dalessandro, G , De Santis, A , and Arrigoni, O , *Pl Cell Physiol*, **14**, 487–496 (1973)
5 Liso, R , and Calabrese, G , *Phycologia*, **14**, 1–4 (1975).
6 Wagner, G., and Loewris, A , *Pl Physiol*, *Lancaster*, **54**, 784–787 (1974)
7 Smith, F S , *Pl Physiol*, *Lancaster*, **27**, 736–744 (1952)
8 Mineshita, T , Yamaguchi, K , Takeda, K , and Kotera, K , *A Rep Shionogi Res Lab*, **6**, 119–130 (1956)
9 Mineshita, T., Yamaguchi, K , Takeda, K , and Kotera, K., *A. Rep Shionogi Res Lab*, **6**, 131–141 (1956)
10 Roe, J. H , and Oesterling, M J , *J biol Chem*, **152**, 511–521 (1944)
11 Liso, R , and Calabrese, G , *Phycologia*, **13**, 205–208 (1974).
12 Hackett, D. P , Haas, D. W , Griffiths, S H., and Niederpruem, D J , *Pl Physiol.*, *Lancaster*, **35**, 8–19 (1961)
13 Boit, H G , *Chem Ber*, **87**, 1704 (1954)

Intracellular killing of *Listeria monocytogenes* by activated macrophages (Mackaness system) is due to antibiotic

THE classic *in vivo* studies of Mackaness[1] showed that the macrophage could be activated to kill intracellular organisms such as *Listeria monocytogenes*. This led to a series of experiments to determine the process by studying killing of this organism by macrophages both *in vivo* and *in vitro*[2-6]. Subsequent experiments carried out with Mackaness[1] system *in vitro* have required antibiotic or suitable serum in the culture medium to control extracellular growth of the organism which otherwise overruns the culture[7,8]. Antibiotics have been shown
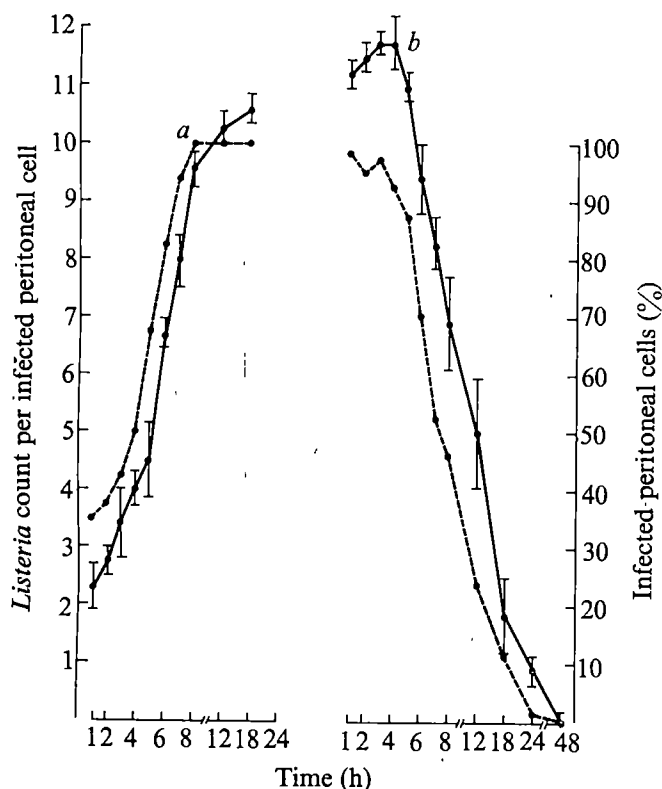
Fig. 1 The effect of antibiotic on microscopically visible avirulent intracellular *Listeria monocytogenes*. Glass-adherent cells from peritoneal washings of 10-week-old, female BALB/c mice were infected with an avirulent derivative of the *L. monocytogenes* strain NCTC5214 at a multiplicity of infection of 10. Cultures were washed and incubated in medium 199 with 20% heat-inactivated foetal calf serum in the absence (*a*) or presence of 0.8 μg ml⁻¹ benzyl penicillin (*b*). ●———●, Avirulent *Listeria* count per infected peritoneal cell (left-hand vertical axis scale); ● ─ ─ ─ ●, % infected peritoneal cells (right-hand vertical axis scale). Each point represents the mean ± s.d of triplicate cultures in which 100 peritoneal cells were counted.

Fig. 2 The effect of antibiotic on peritoneal cell survival in cultures infected with avirulent *Listeria*. ●———●, Uninfected culture without antibiotic; □———□, *Listeria*-infected culture without antibiotic; ○———○, *Listeria*-infected culture with 0.8 μg ml⁻¹ penicillin. Each point represents the mean of triplicate cultures in which 100 high power fields were examined.

In the absence of antibiotic in the medium, apparent 'proliferation' of avirulent *L. monocytogenes* within the peritoneal cells occurs (Fig. 1) and the culture undergoes premature destruction (Fig. 2). To preserve the cell culture it is necessary

Fig. 3 Microscopically visible intracellular *Listeria* in cultures with antibiotic (benzyl penicillin 0.8 μg ml⁻¹). ■, *Listeria* strain NCTC5214 of intermediate virulence; ▼, virulent derivative of *Listeria* NCTC5214; ———, *Listeria* count per infected peritoneal cell (left-hand vertical axis scale); ─ ─ ─, % infected peritoneal cells (right-hand vertical axis scale). Each point represents the mean ± s.d. of triplicate cultures in which 100 peritoneal cells were counted.

to enter peritoneal cells[9] and affect bacterial proliferation[10-12], although there is evidence that the intracellular environment to some extent protects bacteria against the effects of antibiotics[13,14]. It has always been uncertain, therefore, whether any intracellular killing observed has been a result of antibiotic rather than activated cellular processes. Furthermore, antibiotic might be expected to enter activated cells more than resting ones, thereby causing an apparently increased intracellular killing by the former. Thus, in order to validate the system, it is necessary to prove that antibiotic is not involved in the killing of the organism. The crucial, but hitherto absent, control that is required must show true intracellular proliferation of the organism within activated cells in the face of antibiotic in the medium.

Fig. 4 Viable intracellular *Listeria* in normal and activated peritoneal cultures in the presence of antibiotic. Activated peritoneal cells consisted of glass-adherent peritoneal exudate cells induced by 2 ml of 10% proteose–peptone i.p. for 72 h. Viability assay, read at 36 h, was carried out by pour-plate dilution method in tryptose–soya agar after the cultures had been scraped with a rubber policeman, the cells lysed in distilled water and the lysate sonicated. Triplicate assays were carried out from each culture. ●———●, Avirulent *Listeria* within normal peritoneal cells; ■———■, intermediate-virulence *Listeria* (NCTC 5214) within normal peritoneal cells; ▼———▼, virulent *Listeria* within normal peritoneal cells; ◆ – – – ◆, virulent *Listeria* within activated peritoneal cells. Each point represents the mean ± s.d. of triplicate cultures.

to add antibiotic (penicillin at twice the minimal inhibitory concentration (MIC) for the organism) to the culture medium, thereby suppressing extracellular growth of the organism. This results in quite the reverse of intracellular proliferation, for both microscopy (Fig. 1) and viable intracellular counts (Fig. 4) indicate that death of the organism occurs within the cells—raising the question of whether this killing is due to antibiotic penetration of the peritoneal cell.

There is evidence for penetration of antibiotic into peritoneal cells[9] and even for concentration of it within such cells[15]. It could cause *Listeria* killing within the cells in two ways. Either it could act bactericidally or it could prevent proliferation of the organism (bacteriostasis), enabling the peritoneal cells to do the actual killing. The only way to show that antibiotic does not act in these ways is to demonstrate a proper control of genuine intracellular proliferation of *L. monocytogenes* in the face of such antibiotic. Studies of strain NCTC5214 of intermediate virulence did not resolve the question. In the absence of antibiotics, strain NCTC5214 behaved identically to the avirulent derivative, but on addition of antibiotics it persisted in a viable state within the cells (Figs 3 and 4) instead of being destroyed. Nevertheless, it did not proliferate and so was not a proper control.

Proof of the ability of *L. monocytogenes* to undergo true

intracellular proliferation in the face of antibiotic in the medium was furnished by a series of experiments using a more virulent strain derived from NCTC5214 by modification of the technique of continuous passage in mice. Using this strain it was evident from microscopy (Fig. 3) and viability assay (Fig. 4) that true proliferation had occurred. Furthermore, as the MIC of antibiotic for all three strains of organism was identical (0.4 µg ml⁻¹) the different behaviour of the three strains within the peritoneal cells cannot be explained by the effects of antibiotic alone.

When peritoneal cells are activated by various methods their permeability to antibiotic may increase and therefore enable greater intracellular concentrations to occur. Thus, the apparent killing of intracellular organisms in the activated cell may still be due to antibiotic rather than true cellular killing.

Repeating the experiment with activated peritoneal cells and using the same three strains of *L. monocytogenes* resolved the problem. This time the behaviour of the organisms was different — the avirulent strain was killed more rapidly, the NCTC5214 strain was killed (whereas in normal peritoneal cells it had remained in stationary phase), and the virulent strain proliferated, but at a slower rate than in non-activated peritoneal cells (Fig. 4). Again, as the MIC of penicillin for these strains was identical, the increased rates of killing of the avirulent derivative and NCTC5214 strain and the slowed proliferation of the virulent strain must have been a result of factors unrelated to the antibiotic permeability of the peritoneal cells.

A dose–response experiment (collected after 18 h culture) showed that if a penicillin concentration of 1.2 µg ml⁻¹ or more were achieved in the extracellular medium the virulent strain of *L. monocytogenes* was killed intracellularly. This confirms that penicillin enters the cells and can have a bactericidal effect—making a proper control mandatory.

A further problem in the interpretation of experiments previously reported is the fact that during the first 4 h of culture

Fig. 5 Dose–response effect of antibiotic on intracellular proliferation of the virulent derivative of *Listeria* after an 18 h culture. Each point represents the mean ± s.d. of triplicate cultures
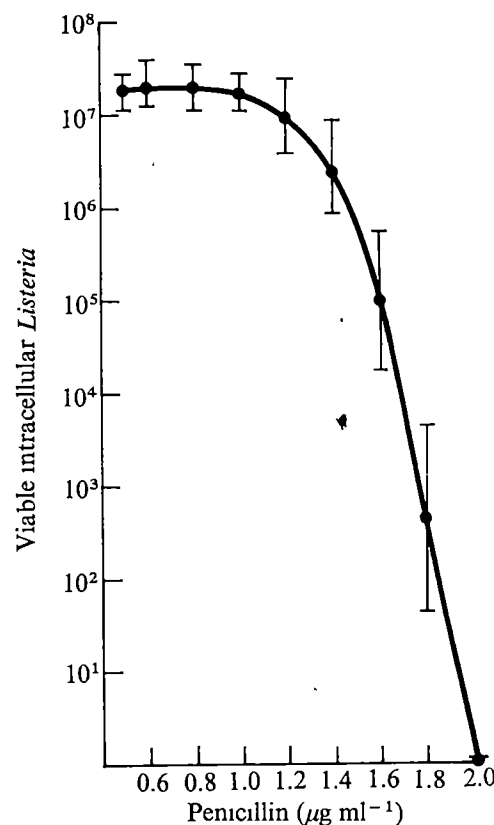
there is an 'apparent' increase in the microscopical counts of bacteria (Fig. 3) within peritoneal cells in spite of the presence of antibiotic in the medium. The viable counts (Fig. 4) do not reflect this; therefore this apparent increase is due to the phagocytosis of dead or dying organisms from the extracellular medium in the period following addition of the antibiotic at the end of the infecting phase. If only short term cultures are carried out, without close attention to viable counts of intracellular organisms, a misleading impression of intracellular proliferation may be gained.

We have shown that omission of antibiotic, because it enables uncontrolled extracellular proliferation of the organism, results in misleading data indicating more exuberant intracellular proliferation of *L. monocytogenes* than is in fact occurring. On the other hand our data show that concentrations of penicillin of 1.2 μg ml^{-1} or more kill the strain of *L. monocytogenes* used within activated mouse macrophages. As the MIC of penicillin for all sensitive strains of *L. monocytogenes* tested to date has been less than 0.5 μg ml^{-1}, it is highly likely that the critical concentration of penicillin in the culture medium for most strains of this organism in this system approximates to 1.2 μg ml^{-1}; but it is likely also to depend on the kind of macrophages used and (or) their level of activation. Where experiments have been carried out using higher concentrations of antibiotic it is doubtful whether activation of the macrophages *per se* has contributed to the observed killing.

The necessary control consists of true intracellular proliferation of the organism in the face of antibiotic in the medium. We have found that concentrations of penicillin less than 1.2 μg ml^{-1} enable both the intracellular proliferation of *L. monocytogenes* and the demonstration of the bactericidal property of activated macrophages.

This work was supported by a Wellcome Trust grant.

PETER COLE*
JONATHAN BROSTOFF

Division of Communicable Diseases,
Clinical Research Centre, Harrow
and Department of Immunology,
Middlesex Hospital Medical School,
London W1, UK

*Present address and address for reprints· Host Defence Unit, Department of Medicine, Cardiothoracic Institute, Fulham Road, London SW3, UK.

1 Mackaness, G. B., *J. exp. Med.*, 116, 381–406 (1962).
2 Mackaness, G. B., *J. exp Med*, 120, 105–120 (1964).
3 Blanden, R. V, Mackaness, G. B., and Collins, F. M., *J exp. Med*, 124, 585–600 (1966).
4 Blanden, R. V., *J. reticuloendothel. Soc.*, 5, 179–202 (1968).
5 Mackaness, G B, *J. exp. Med.*, 129, 973–992 (1969)
6 McGregor, D D, Koster, F. T., and Mackaness, G. B., *J exp. Med*, 133, 389–399 (1971).
7 Wilder, M S, and Edberg, J. C, *Infect. Immun.*, 7, 409–415, (1973)
8 Rhodes, M W., and Hsu, H. S., *J reticuloendothel. Soc.*, 15, 1–12 (1974)
9 Jenkins, C. R., and Benacerraf, B., *J exp. Med*, 112, 403–417 (1960).
10 Patterson, R J, and Youmans, G. P., *Infect. Immun.*, 1, 30–40 (1970).
11 Simon, H. B., and Sheagren, J. N., *J exp Med*, 133, 1377–1389 (1971).
12 Adam, D, Staber, F., Belohradsky, X., and Marget, W., *Infect. Immun*, 5, 537–541 (1972).
13 Magoffin, R. L., and Spink, W. W, *J Lab. clin Med*, 37, 924–930 (1951).
14 Shaffer, J. M, Kucera, C. J., and Spink, W. W., *J. exp Med.*, 97, 77–89 (1953).
15 Bonventre, P F., and Imhoff, J. G., *Infect. Immun.*, 2, 89–95 (1970).

Electron spin resonance measurements of molecular interactions in mouse olfactory epithelium

THE mechanism by which molecules interact with, and so stimulate, the surfaces of olfactory receptor cells has given rise to much speculation. Since oil-soluble substances are often very effective odours, it has been proposed that olfactory transduction occurs when an odorant molecule perturbs the lipids of the sensory membranes. Conversely, the known steric aspects of odorant molecule interactions argue for a more specific binding for which a protein would be a more suitable receptor[1–4].

These theories are based mainly on inferences of how odorants of known chemical structure should behave in biological membranes but there is a lack of direct measurements on olfactory tissue in support of either view. Here we report the use of electron spin resonance (ESR) spectroscopy using spin-labelling techniques[5,6] to investigate the molecular interactions of odorants at olfactory and other membrane surfaces.

Solutions of spin-labelled nitroxide compounds in saline or Ringer's (10^{-3}–10^{-5} M) were injected into the nasal chamber of mice immediately after death by cervical dislocation or an overdose of Nembutal, and after 3–5 min olfactory tissue was removed, and placed in saline or Ringer's in an ESR tissue cell. The spectra were measured on a Varian E9 ESR spectrometer operating at the X band. Long scan times were essential to resolve the spectra. Respiratory mucosa from the nasal chamber and mucosa from the small intestine were used as tissue controls.

In the case of the four odourless spin-labelled compounds investigated using the olfactory epithelium (Fig. 1),
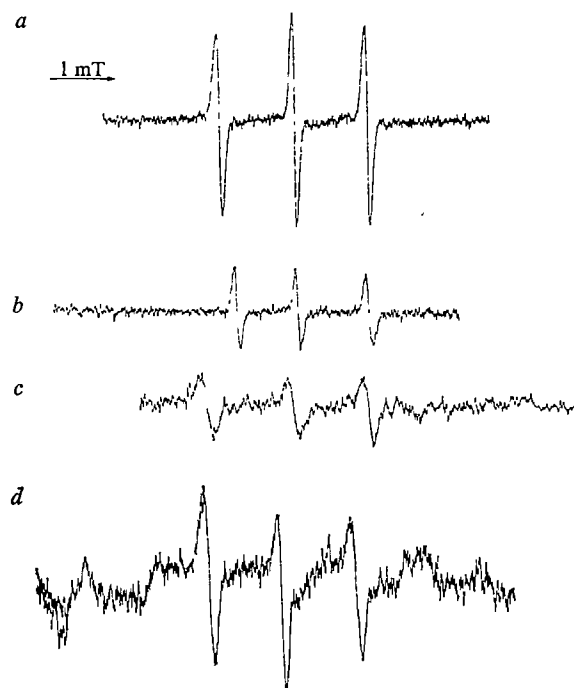
Fig. 1 ESR spectra of four odourless spin labels in the mouse olfactory epithelium. *a*, 3-Carbamoyl-2,2,5,5-tetramethylpyrrolinooxyl; *b*, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidinooxyl; *c*, 4-acetylamino-2,2,6,6-tetramethylpiperidinooxyl; *d*, 4-hydroxy-2,2,6,6-tetramethylpiperidinooxyl.

the three amides showed spectra characteristic of their presence in an aqueous environment; since there is no evidence of molecular association with the cell membranes they are therefore assumed to remain in the mucus layer. The spectrum of the fourth spin-labelled compound (4-hydroxy) shows a splitting of the high field line (characteristic of the partitioning of a spin label between aqueous and lipid phases), and in addition a weak, strongly immobilised component.

Most of the odorous spin labels give partially immobilised spectra indicative of their solution in the membrane lipid (Fig. 2), whereas, of those compounds remaining (also the strongest odours), the isothiocyanate and *p*-toluene sulphonate ester derivatives are strongly immobilised (Fig. 3*a* and *b*), and the 4-piperidone shows a strongly immobilised component ($g=2.008$ and 1.999; Fig. 3*c* and *d*). Isothiocyanates readily react with free primary amino, thiol or hydroxyl groups, and in this instance, the isothiocyanate is probably so bound to mem-

brane proteins. The spin labels of each class gave spectra with the control tissues which were similar to those with the olfactory epithelium.

Previous experiments have shown a good general correlation of the odour thresholds of various mammals and of man although they cannot be claimed to be identical[7].

Since the spectra of sensory and non-sensory tissues were closely similar it does not seem that any special features of the olfactory epithelium can be detected with this technique, but we can in broad terms draw a parallel between the olfactory potency of a compound and the extent to which it interacts with the lipids and other components of cellular membranes in general.

Recent experiments on frogs[8,9] indicate that receptor sites on olfactory endings consist at least in part of proteins. We suggest that such proteins may be buried in the lipid of the olfactory membranes or sited at their inner surface so that an odour–lipid interaction is needed before an odour can reach the receptor site. This inaccessibility would provide a greater degree of biological specificity.

In the case of the strongest odours, a strong interaction, in some instances chemical bond formation, probably occurs between odour and membrane protein, such as to pro-

Fig. 2 ESR spectra of five odourous spin labels in the mouse olfactory epithelium. *a*, 4-Amino-2,2,6,6-tetramethylpiperidino-oxyl; *b*, 4-isocyano-2,2,6,6-tetramethylpiperdinooxyl; *c*, 4-acetyloxy-2,2,6,6-tetramethylpiperidinooxyl; *d*, 4-benzyloxy-2,2,6,6-tetramethylpiperidinooxyl; *e*, 2,2,6,6-tetramethylpiperidinooxyl.
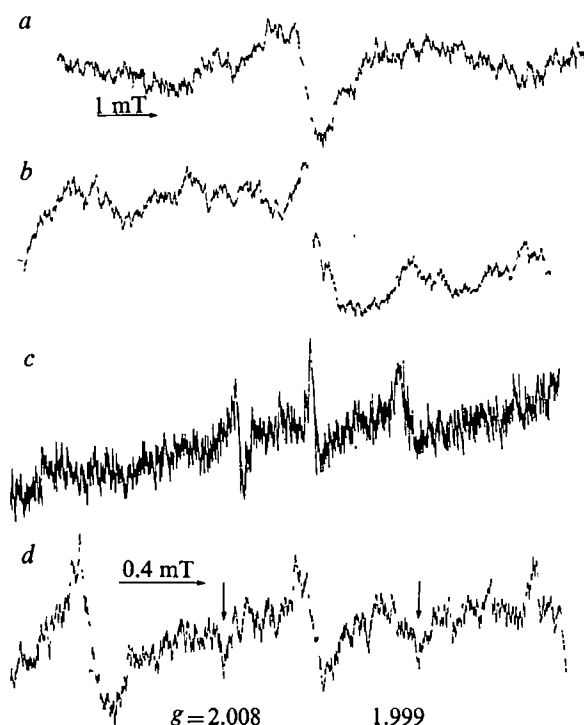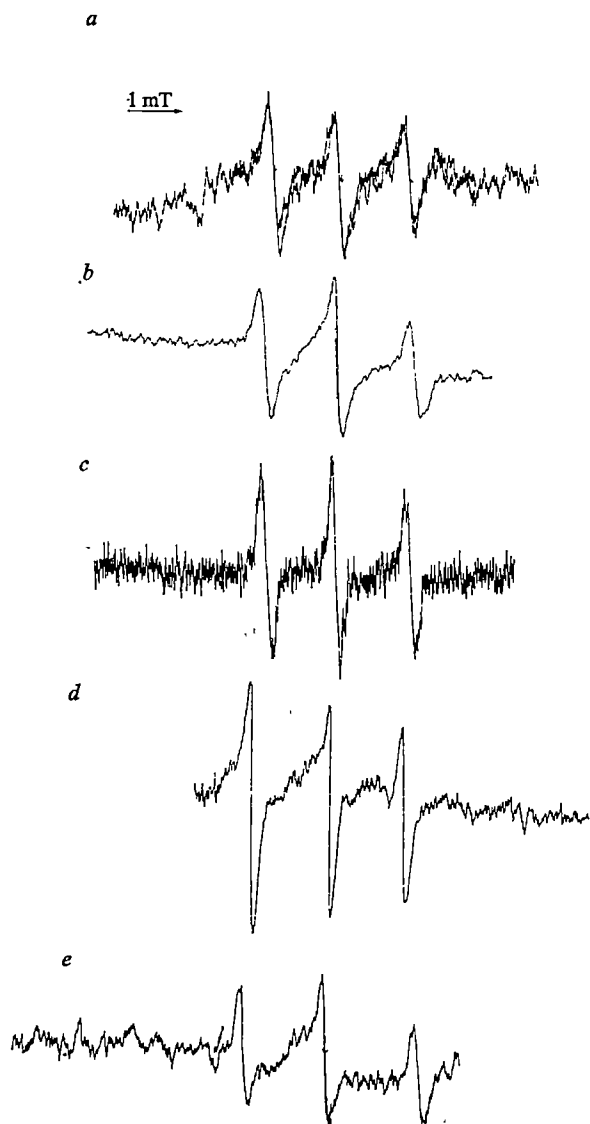
Fig. 3 ESR spectra of three pungent spin labels in the mouse olfactory epithelium. *a*, 2,2,6,6-Tetramethyl-4(4'-toluene sulphonate) piperidinooxyl; *b*, 4-isothiocyanato-2,2,6,6-tetramethylpiperidinooxyl; *c*, and *d*, 4-oxo-2,2,6,6-tetramethylpiperidinooxyl.

mote a conformational change, and so initiate olfactory transduction. Similar mechanisms may operate for the less reactive but lipophilic odours. The absence of appreciable odour in the case of the 4-hydroxy compound suggests, however, that penetration of the membrane is not in itself a sufficient condition for stimulation. This evidence therefore argues against a purely lipid–odour interaction in olfaction. Further experiments are in progress to test these conclusions, and a theoretical treatment of these results, in terms of current theories of cooperative phenomena in membranes, is in preparation.

L. H. Bannister

Department of Biology,

A. D. Clark
L. J. Dunne
S. J. Wyard

Division of Biological Sciences,
Department of Physics,
Guy's Hospital Medical School,
University of London,
London Bridge, London SE1 9RT, UK

1 Davies, J. T., in *Handbook of Sensory Physiology*, 4 (edit. by Beidler, L. M.), part 1, 322 (Springer, New York, 1971)
2 Amoore, J. E., in *Taste and Smell in Vertebrates* (*CIBA Foundation Symposium*) (edit. by Wostenholme, G. E. W., and Knight, J.), 293 (Churchill, London, 1970).
3 Mazziotti, A., *Nature*, 250, 645 (1974)
4 Wright, R. H., *Nature*, 209, 571 (1966).
5 McConnell, H. M., and McFarland, B. G., *Q.Rev. Biophys.*, 3, 91 (1970).
6 Roubal, W. T., *Prog. Chem. Fats*, 13, 63 (1972).
7 Moulton, D. G., Celebi, G., and Fink, R. P, in *Taste and Smell in Vertebrates* (*CIBA Foundation Symposium*), (edit. by Wolstenholme, G. E. W., and Knight, J.), 227–250 (Churchill, London, 1970).
8 Getchell, M. L., Gesteland, R. C., *Proc. natn Acad. Sci. U.S A.*, 69, 1494 (1972).
9 Getchell, T. U, Getchell, M. L., *Ann. N.Y. Acad Sci*, 237, 62 (1974).

matters arising

The design of wildlife preserves

MAY[1] has drawn attention to the need to make nature reserves sufficiently large, arguing from both theory and observations on island biogeography I would like to add two comments to reinforce his case. The first is that a similar species–area relationship is found in quadrats of different size and in other studies of continuous ecosystems, as well as across sets of islands. This means that when a boundary is drawn around a nature reserve in a larger area of the same habitat, the number of species in the reserve will be less than that in the region as a whole. The other point is that if the remainder of the habitat is then cleared away, there will be an edge effect around the new reserve. Species suited to the edge will frequently be different from those suited to the centre of the reserve. If the reserve is intended to maintain the 'central' species, then its effective size is smaller than its apparent area.

MARK WILLIAMSON

Department of Biology,
University of York, York YO1 5DD, UK
[1] May, R. M , *Nature*, 254, 177–178 (1975).

Sudden death in infancy

I AM pleased to learn that Carpenter and Emery[1] have succeeded in identifying a limited number of high-risk cases in the field of child-death prevention. But their conclusion that "there are encouraging indications that the study may be preventing some deaths" is not supported by the data.

Carpenter and Emery[1] compared a high-risk group which was not selected for follow-up health care with a sample of a low-risk group, and found that the former had significantly higher mortality. But this result merely reflects the original group assignments. The relevant issue is rather, whether or not the high-risk group which was followed up differs from the high-risk group which was not selected.

I have reanalysed Carpenter and Emery's data (Table 1) using χ-square tests corrected for continuity. Two groups (high-risk subjects not selected, and high-risk subjects either not selected or not participating) are compared separately with the high-risk group receiving aftercare. The null hypothesis for each test is that mortality will be at least as great in the follow-up group as in the non-treatment group. As statistical convention requires significance at the 0.05 level, the null hypothesis cannot be rejected in either case.

Perhaps a larger sample will yield different results in the future.

STEPHEN MAGURA
New Jersey Division of Youth and Family
Services,
1 S. Montgomery Street,
Trenton, New Jersey 08625

[1] Carpenter, R G , and Emery, J L., *Nature*, 250, 729 (1974)

CARPENTER AND EMERY REPLY—

Magura[1] is mistaken when he says that the significant difference we reported[2] "merely reflects the original group assignments". The group assignments were based on a discriminant function that had been constructed before the prospective study began. The significant difference referred to is fundamental to our main point, which is that it is possible to identify babies who will be at risk of sudden death in infancy within 24 hours of their birth. We also showed that the high-risk group is sufficiently sharply defined that it can be studied prospectively.

Magura shows that the observed difference in mortality rates between the high-risk group followed up and the high-risk controls is compatible with the null hypothesis, as we were well aware. But the data are equally compatible with the alternative hypothesis that the follow-up study is preventing deaths, and on a likelihood-ratio criterion the latter hypothesis is more strongly supported than the

former. This common sense view is also reinforced by case reports[3]. We think that our conclusion that "there are encouraging indications that the study may be preventing some deaths" summarised this situation accurately.

We are confronted with a difficult ethical problem. To know whether or not the discriminant function and the follow-up study are effective a high-risk control group is essential. But in the light of results such as those summarised by Magura, for how long does it remain ethical to exclude high-risk babies from the follow-up study? The classical significance test approach followed by Magura gives one answer to the problem. It would be interesting to know if this view is generally shared by your readers.

London School of Hygiene
and Tropical Medicine,
and The Children's Hospital,
Western Bank, Sheffield 10, UK

[1] Magura, S , *Nature*, 256, 519 (1975)
[2] Carpenter, R. G , and Emery, J. L , *Nature*, 250, 729 (1974)
[3] Emery, J. L , and Carpenter, R. G , *SIDS* 1974 (edit. by Robinson, R. R ,) 97-106 (Canadian Foundation for the Study of Infant Deaths, Toronto and London, 1974).

Proterozoic supercontinent: time duration and the Grenville problem

THE reconstruction of the Proterozoic supercontinent has been devised[1] by superimposing 2,000–1,100 Myr palaeomagnetic poles from two regions and was derived by rotating North America anticlockwise 146° about a Euler pole at 138°E, 73°N. With this operation most dated, Precambrian, palaeomagnetic poles from Africa correlate with comparable poles from North America with fields of agreement illustrated in a qualitative way in Fig. 1. The new data of McGlynn *et al.*[2] enable further comparison and the 2,090 Myr Indin dykes pole agrees closely with several poles 2,070–2,090 Myr in age from Africa[3]. Also, 2,150 Myr poles from western Africa correlate with some poles from the Nispissing diabase and Abitibi dykes (~2,150 Myr). It is not yet possible to match 2,200–2,300 Myr poles from Africa with North American data, and there are clearly two possibilities: either the reconstruction is not valid earlier than 2,150 Myr or apparent polar movements are more complicated than recognised.

The latter explanation is probably

Table 1 Number of infants and number of sudden deaths among high-risk groups

	No. in group	No of deaths	Significance (one-tailed test)
Followed up	354	0	
Not selected	477	4	$0.15 > P > 0.10$
Not selected or not participating	557	5	$0.10 > P > 0.05$

correct for three reasons. First, there is general agreement between pre-2,400 Myr poles on the supercontinent reconstruction (Fig. 1). This grouping seems to be definitive: the new 2,692 Myr pole from the Dogrib dykes of North America[4] falls in the same group and close to the 2,630 Myr pole from the Modipe gabbro of Botswana. Second, palaeomagnetic studies of the ~2,160 Myr Nispissing diabase and Abitibi dykes yield discordant poles defining similar tracks[2], implying rapid apparent polar movement at the time of their intrusion. Third, Archaean foliated anorthosites seem to occupy a single linear belt[5,6] on the reconstruction.

The Proterozoic supercontinent reconstruction reconciles what has become one of the most intractable problems of North American Precambrian palaeomagnetism. Pole positions from rock units within the Grenville Province form a group practically distinct from pole positions derived from elsewhere in the Canadian Shield[7]. It is argued that the Grenville poles are essentially contemporaneous with quite different pole positions from rock units north of the Grenville Front[7,8] and that they may be explained in terms of plate collision between the Grenville Province and the remainder of the shield at about 1,000 Myr. This explanation is difficult to reconcile with the protracted history of the province and the correlation of features across the line of the Grenville Front well into the Grenville Province (see ref. 9). The plate tectonic hypothesis has become virtually untenable in the light of new palaeomagnetic work[10] yielding Grenville-type poles from a region close to the Grenville Front where there seems to be little possibility of a plate suture between the province and the shield, and also from identification of Grenville-type palaeomagnetic directions within the Canadian Shield and remote from the Province[11].

In Fig. 2 the Grenville Province poles from the compilation of ref. 7 are plotted on the supercontinent reconstruction where it is seen that they straddle the apparent polar wander curve recognised from African data between about 1,040 and 800 Myr consistent with abundant radiometric data from the province. It implies that the Grenville Province was an integral part of the Proterozoic supercontinent; it also suggests that this supercontinent existed as such to about 800 Myr BP.

Fig. 1 Fields of agreement between African and North American Proterozoic palaeomagnetic poles on the supercontinent reconstruction. Figures give ages in millions of years. The <1,730 Myr field is defined by redating of the Mackenzie III dykes[12]. Pre-2,400 Myr poles from Africa (squares) and North America (circles) are plotted and their ages indicated in millions of years.
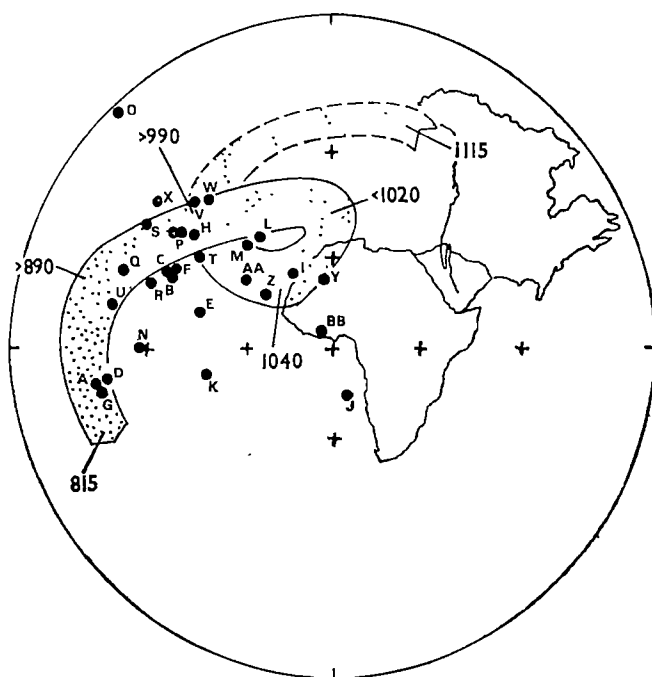
Fig. 2 Pole positions from rock units within the Grenville Province of North America plotted on the supercontinent reconstruction. The apparent polar wander path derived from African data between 1,115 and 800 Myr BP is also indicated by the stippled swathe (refs 1 and 3); this path is derived in part from stratigraphically-controlled series of poles and the figures are assigned ages in millions of years. The poles are: A, Allard lake; B, Frontenac dykes; C, Grenville gneisses; D and E, Haliburton basic rocks; F, Lake St Jean anorthosite; G and H, Morin anorthosite, I, Morin dykes; J, Magnetawan metasediments; K, L, and M, Mealy Mount; N, Larrimac and Bryson diorites; O, Tudor gabbro; P, Unfraville intrusive; Q, Wilberforce pyroxenites, R, S and T, Whitestone anorthosite; U, Whitestone diorite; V, Grenville front anorthosites; W, Michael gabbro; X, Seal and Croteau igneous; Y, Seal group red beds; Z, AA and BB, Shabogamo gabbro.

J. D. A. PIPER

Sub-department of Geophysics,
University of Liverpool,
Liverpool L69 3BX, UK

1 Piper, J D A , *Nature*, 251, 381 (1974)
2 McGlynn, J C , Irving, E., Bell, K , and Pullaiah, G., *Nature*, 255, 318 (1975)
3 Piper, J D A , Briden, J. C , and Lomax, K., *Nature*, 245, 244 (1973)
4 McGlynn, J. C , and Irving, E , *Tectonophysics*, 262, 26 (1975)
5 Windley, B F , and Bridgewater, D , *Spec. Publ. geol. Soc Aust* , 3, 33 (1971).
6 Sutton, J , in *Implication of Continental Drift to the Earth Sciences* (edit. by Tarling, D. H., and Runcorn, S K), 1071 (Academic, London, 1973)
7 Irving, E., Emslie, R F , and Ueno, H , *J. geophys Res* , 79, 5491 (1974)
8 Irving, E., Park, J. K , and Roy, J. L , *Nature*, 236, 344 (1973)
9 Windley, B F , *Proc R Soc* , 273, 389 (1973).
10 McWilliams, M O , and Dunlop, D J , *Trans. Am. geophys Un.*, 55 (4), 226 (1974).
11 Halls, H C , *Nature*, 255, 692 (1975).
12 Gates, T M , and Hurley, P M , *Can. J Earth Sci* , 10, 900 (1973)

Bottom life under Antarctic ice shelves

WITH reference to the article by Heywood and Light[1], it should be pointed out that the existence of a relatively rich and diversified benthic fauna under an Antarctic ice shelf was first demonstrated by Littlepage and Pearse[2] in 1962 for the Ross ice shelf During November and December 1961 they collected various representatives of 16 major zoological groups (including the fish *Trematomus* sp) using traps and grabs inserted through cracks in the shelf ice at distances of 22 and 28 km from the open sea

As those samples included some typical 'suspension-feeders' (Porifera, Ectoprocta, Sabellida and so on), a water current able to transport the food items evidently exists and may explain the development of a rich bottom fauna under the Ross Ice Shelf. No doubt, such a current is also present under the Shelf ice of King George VI Sound, according to the shape and two openings of this ice covered body of water

In fact, I see no reason why a substantial bottom fauna should not live anywhere under any floating Antarctic ice shelf, as many Antarctic invertebrates and fish are adapted to an opportunistic diet (including even necrophagy)[3]. Furthermore, there is no longer any reason to expect a peculiar (specific) fauna under Antarctic ice shelves, as it is now admitted that the Antarctic shelf has been covered by the edges of the Wurmian ice sheet. So, the main biological interest of the Ross Ice Shelf Project (RISP), when drilling at a distance of 450 km from the seaward edge of the Ross Ice Shelf, would be to obtain an insight into adaptations and relationships among the biota of these obscured areas of the Antarctic shelf.

PATRICK M. ARNAUD
Station marine d'Endoume,
Rue de la Batterie des Lions,
13007 Marseille, France

[1] Heywood, R B., and Light, J J , *Nature*, 254, 591 (1975)
[2] Littlepage, J L , Pearse, J. S , *Science*, 137, 679 (1962)
[3] Arnaud, P M , *Third Symposium on Antarctic Biology, Washington* (in the press)

HEYWOOD AND LIGHT REPLY—We are grateful to Arnaud for drawing our attention to the paper by Littlepage and Pearse, an unfortunate oversight on our part We note from this paper the *Trematomus* sp. was found by DeVries and Kooyman We agree of course with the ecological comment of Arnaud. We prefer, however, to keep an open mind on whether a substantial bottom fauna can live anywhere under a floating Antarctic

ice shelf—the problems of obtaining enough food at a distance of 450 km from the open sea could be far greater than when merely 28 km away. We believe this justifies our remarks that the first biological aim of RISP is to determine whether a biome can exist at a considerable distance from the open sea, and that a biome found under ice 100–500 m thick, at least 100 km from the open sea, is " remarkable ".

Monks Wood Experimental Station, Abbots Ripton, UK

Random packing of equal spheres

THE recent article of Gotoh and Finney[1] has drawn my attention to this interesting problem. I have been impressed by the amount of experimental work carried

Fig. 1 Packing of circles and triangles.

on in this field, but at the same time I am wondering why similar experimental work has not been done—as far as I know—on the theoretically simpler problem in two dimensions to provide some evidence. I have also noticed several more or less explicit pleas to mathematicians to 'invent a statistical geometry'. None of the authors seems aware that such a geometry does indeed exist. It is usually called "integral geometry" (see, for example, ref. 2) and its methods should certainly be relevant to this problem.

The origin of integral geometry can be traced back to the famous 'Buffon's needle' problem. A needle of length *l* is thrown on a board ruled with parallel lines at distance *d*. One can show that the average number *n* of contacts of the needle with the lines is

$$n = (2/\pi)(l/d) \qquad (1)$$

Integral geometry has several theorems such as (1) in which the constant $2/\pi = 0 6366197 \ldots$ appears. Could this be the "maximum packing density" given in ref. 1 as 0.6366 ± 0.0008 and 0.6366 ± 0.0004 in two different experiments? It is certainly a conjecture worth pursuing.

In the meantime I would like to suggest an elementary explanation of the random loose packing density Let us start from the two-dimensional case.

Circles and triangles take the place of spheres and tetrahedra. In the language of Gotoh and Finney we have a triangle —formed by an arbitrary circle and two supporting circles—which is completely specified by the angle *a* (see Fig. 1). To compute the packing density it is convenient to add a circle on the bottom to make the drawing more symmetrical. We can then easily see that the packing density of the configuration is the area of one circle divided by the (hatched) area of the parallelogram. Assuming a uniform probability density for the angle *a*, the average area *A* of the parallelogram is then

$$A = \frac{\int_{\pi/3}^{\pi/2} 4r^2 \sin a \, da}{\int_{\pi/3}^{\pi/2} da} = (12/\pi)r^2 \qquad (2)$$

The limit $\pi/3$ corresponds to the case of the two supporting circles in contact; angles greater than $\pi/2$ give a configuration equivalent to one rotated by 90°. The average packing density *d* is then

$$d = (\pi r^2/A) = (\pi^2/12) \sim 0.8225 \qquad (3)$$

All configurations being taken as equally likely with no correlations this density
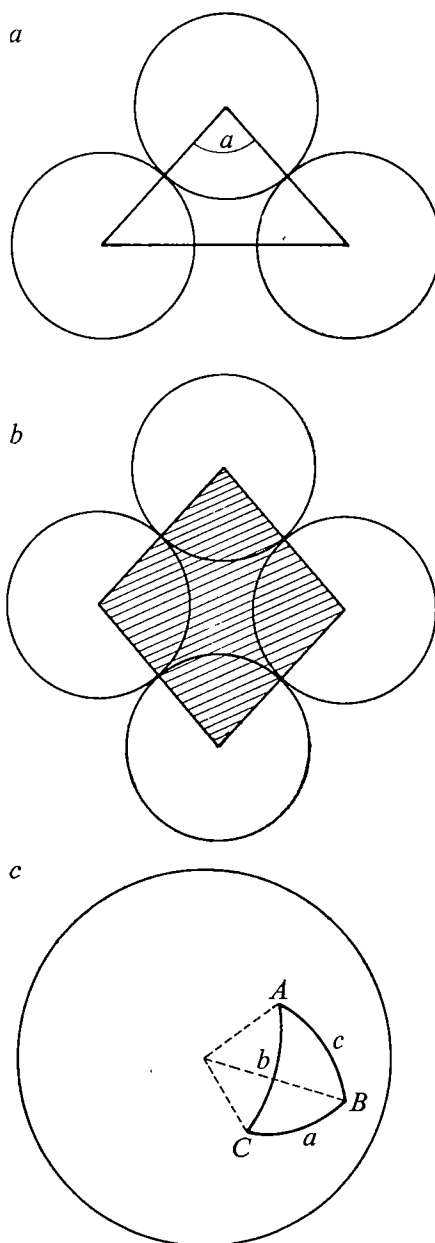
should correspond to the case of random loose packing of equal circles. The three-dimensional problem can be solved in a similar way. Figure 1 should be changed into the Gotoh and Finney spheres and tetrahedron, and show a hatched parallelepiped. The three vectors from the centre of the supported sphere to the centres of the supporting spheres will then mark the vertices of a spherical triangle on a sphere of radius $2r$ (Fig. 1c). Since the probability of a configuration with b, c, A in the range $dbdcdA$ is proportional to

$$\sin b \ \sin c dbdcdA,$$

using the formula

$$\cos a = \cos b \ \cos c + \sin b \ \sin c \ \cos A$$

one obtains

$$\sin b \ \sin c dbdcdA \qquad (4)$$
$$= (\sin a \ \sin b \ \sin c dadbdc)/U$$

with $U = (1 - \cos^2 a - \cos^2 b - \cos^2 c + 2\cos a \ \cos b \ \cos c)^{1/2}$. The volume of the hatched parallelepiped turns out to be $8r^3 U$.

The average volume of the parallelepiped is therefore (by analogy with equation (2))

$$V = \frac{8 \displaystyle\int_{\pi/3}^{\pi/2} \int_{\pi/3}^{\pi/2} \int_{\pi/3}^{\pi/2} \sin a \ \sin b \ \sin c dadbdc}{\displaystyle\int_{\pi/3}^{\pi/2} \int_{\pi/3}^{\pi/2} \int_{\pi/3}^{\pi/2} \sin a \ \sin b \ \sin c dadbdc/U} \qquad (5)$$

With a simple change of variables ($x = \cos a$, $y = \cos b$, $z = \cos c$) we get for the density d (by analogy with equation (3))

$$d = (4/3)\pi \int_0^{1/2} \int_0^{1/2} \int_0^{1/2} dxdydz/(1 - x^2 - y^2 - z^2 + 2xyz)^{1/2} \qquad (6)$$

With a numerical integration I found $d \sim 0.596$, in excellent agreement with the experimental random loose packing density.

A. GAMBA
Istituto di Fisica dell 'Università,
Viale Benedetto XV, 5
Genova, Italy

1 Gotoh, K., and Finney, J. L., *Nature*, 252, 202 (1974).
2 Santaló, L. A., *Introduction to Integral Geometry* (Hermann, Paris, 1953).

GOTOH AND FINNEY REPLY—We are aware of the numerical closeness of $2/\pi$ and 0.6366, but until now had no good nonnumerological reason for asserting its significance. We are grateful to Gamba for pointing out the possible use of integral geometry, and will follow up the references quoted.

We stress the dangers of using two-dimensional analogues[1], for the geometries of two and three dimensions are essentially different. The two-dimensional Ising problem has been solved, although the three-dimensional one has not[2], and the third dimension changes the nature of percolation problems[3]. The average number of edges per Voronoi polygon can be shown to be exactly six for any two-dimensional array of points[4], a fact which blurs the distinction between a two-dimensional "liquid" and crystal. The corresponding three-dimensional case in unsolved, and a discontinuous transition in the number of faces per polyhedron occurs on melting of a three-dimensional liquid[5]. Thus it is difficult to define states in two dimensions that may be equivalent to random loose or close packings in three dimensions[6].

The simplicity of Gamba's explanation of random loose packing density is attractive, but we think it erroneous. In two dimensions, the addition of a fourth circle introduces excessive symmetry, and implicitly assumes that the covering can be considered equivalent to a covering of touching rhombuses. In a real two-dimensional non-crystalline packing, in contrast to a dislocated solid, triangles and polygons occur. Similarly, in the extension to three dimensions, Gamba implicitly assumes that the ran-dom loose packing can be replaced by a space-filling set of touching parallelepipeds. The parallelepiped is an octahedron with two tetrahedral caps, and immediately gives too many octahedra in comparison with the random packing. Moreover, they are distributed in too orderly a manner. The parallelepiped is bounded by six planar faces, each one being defined by four coplanar spheres. This does not fit with the reality of random packing: the extra freedom given by the third dimension leads to a low occurrence of such special arrangements, and a consequent reduction in the local symmetry within the aggregate.

The assumption of a uniform probability density for the angles a, b and c takes no account of the local geometrical restrictions on space occupation (for example, a peak near the icosahedral angle of 65°?) although as we admitted in our original paper, we were unable to take adequate account of these ourselves. We tried to find an "average" density by the admittedly non-rigorous method of finding the "average" dimensions of an "average" tetrahedron We tried to build in the condition that the tetrahedra, of which we try to consider the average, fill space. Thus an integral number meet at an edge, an integral number meet at a vertex, and each face is shared by two tetrahedra. In addition to these basic geometrical constraints, we also considered quasi-physical constraints, such that each sphere should make six contacts on average, and that there should be at least three contacts in any arbitrary hemisphere. Gamba, while he has taken plausible averages, does not take these or equivalent conditions into account. Moreover, by adding a similar unit to the original in a symmetrical manner to facilitate density measurement, he calculates an average over the unit cell of a primitive crystal lattice. It seems to us that his two-dimensional density is a weighted average over all possible unit cells from the square (density 0.785, $a = 90°$) to the hexagonal (density 0.907, $a = 60°$), whereas the three-dimensional value is an average over all possible primitive unit cells from the simple cubic (density 0.524, $a = b = c = 90°$) to the hexagonal and face centred cubic (density 0.704). We do not think this represents random packing

J L. FINNEY
Birkbeck College,
University of London, UK

K GOTOH
University of Hokkaido,
Japan

1 Finney, J L, *Nature*, 242, 398 (1973)
2 Stanley, H E., *Introduction to Phase Transitions and Critical Phenomena*, 17 (Oxford University Press, 1971)
3 Polya, G, *Math Ann*, 84, 149 (1921). Montroll, E W, and Weiss, G H, *J math Phys.*, 6, 167 (1968)
4 Smith, C S, *Metal Interfaces*, 65 (Am Soc Metals, Cleveland, 1965)
5 Finney, J L. *Proc R Soc*, A319, 479 (1970)
6 Quickenden, T. I, and Tan, G K, *J Colloid Interface Sci.*, 48 382 (1974)

Matters arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

reviews

COSMOLOGY is a subject with long traditions, and general relativistic cosmology in particular has been developed in detail, both mathematical and physical, in many textbooks. In recent years there has been a distinct resurgence of interest in the subject, partly resulting from the stimulus of impressive observational discoveries (the microwave background, quasars, and so on) and partly from the elegant new mathematical techniques which have been constructed. Not least among these are the theorems of Hawking, Penrose and Geroch, which predict in very general circumstances that spacetime can develop singularities or 'edges'.

The demand for well written books covering cosmology from this new perspective is growing. Ryan and Shepley have made a useful contribution in this latest volume in the Princeton Series in Physics. An earlier volume by Peebles, called *Physical Cosmology*, deals with observational aspects, whereas this book concentrates on the mathematical side. The authors restrict themselves to models of the Universe which are homogeneous in space. These models, being a good approximation to the large scale structure of the real Universe, are simple enough to allow a tractable mathematical description, but rich enough to confront some of the more challenging and bizarre physical problems.

The early part of the book builds up the terminology and formalism of modern tensor analysis, moving straight into the Robertson–Walker isotropic

Heavenly views
P. C. W. Davies

Homogeneous Relativistic Cosmologies. By Michael P. Ryan Jr and Lawrence C. Shepley. Princeton Series in Physics. Pp. xv+320. (Princeton University Press, Princeton, New Jersey, 1975.) $15.00.

models and the big-bang Friedmann solutions. One of the major themes of the book is then taken up with a fairly detailed description of singularities. Much of this discussion is rather sophisticated, and the new student may feel a certain frustration that very recent developments are not explained in greater detail.

Following the singularities, more traditional work is outlined on the subject of Killing vectors and isometry groups, enabling discussion of the full range of homogeneous cosmological models. Well known examples are described in some detail, though the authors have a tendency to concentrate rather too much on the more bizarre aspects. Godel's model, which apparently allows observers to visit their own past, and the mysterious and baffling Taub-NUT and T-NUT-M spaces, each command a whole section. Some further work on singularities in homogeneous models then follows, leading naturally to the thorny subject of quantum cosmology. In Chapter 10 the authors state that "All explicitly known models which can serve as cosmological models are mathematically

singular", and point out that classical physics runs into a barrier here. The hope that quantum theory will open that barrier is long standing, but current results are still very superficial.

Some of these results are dealt with by Ryan and Shepley. They first build up the idea of 'Hamiltonian cosmology' —treating the Universe as a physical entity moving in a highly simplified fashion with a small number of degrees of freedom only. Quantisation of this simple system is then briefly alluded to. An entire chapter is devoted to the simplest of the anisotropic models, the Bianchi types I and IX, and the so-called mixmaster Universe, in which an initially chaotic motion smooths itself out into the more uniform, isotropic form observed in the real Universe today. A final chapter treats some problems in perturbation theory.

As a teaching textbook, this volume will prove useful. It is inclined to read a little breathlessly, and the level of discussion is somewhat variable, but graduate students and researchers will find it valuable for both reference and instruction. An extensive bibliography is provided, together with some exercises for the more ambitious reader. A little homespun philosophy creeps in here and there, to add a needed touch of perspective on this rather esoteric of subjects.

In summary, this book manages to get to grips with very recent results in cosmology at a time when the subject is undergoing rapid development. It is to be recommended to all serious students of modern cosmology. ☐

HIGH energy astrophysics is a rapidly growing field in which there have been many important discoveries, although graduate students or research workers may find it difficult to obtain a general introduction to the subject, with the possible exception of some lecture notes from summer schools. Now, however, several authors, assoc ated with Nasa's Goddard Space Flight Center, have produced a book which may overcome this problem as far as cosmic X and γ-ray astronomy are concerned. The authors are all well known researchers, each writing about his own specialty.

About one half of the book deals with cosmic rays. Experimental techniques, results concerning nuclear and electron components, and propaga-

tion effects in space are carefully reviewed. Not much is said about sources or about acceleration mechanisms.

The other half of the book deals with X-ray and γ-ray astronomy,

---

High Energy Particles and Quanta in Astrophysics. Edited by Frank B. McDonald and Carl E. Fichtel. Pp. xii+476. (MIT Press: Cambridge, Massachusetts and London, 1974.) $18.50; £9.25.

---

with an introductory chapter on radioastronomy. Little that is basically new has happened in X-ray astronomy over the last couple of years and these chapters can therefore be considered as a good reference. On the other hand, however,

γ-ray astronomy has recently led to the discoveries of bursts and of the SAS 2 results. Since the Goddard Center has been heavily involved in these developments, it is a pity that they were not included.

In a book of this kind a certain amount of bias is unavoidable in the choice and presentation of results. Readers curious about the more general aspects of high energy astrophysics will not find here a discussion of radiogalaxies, quasars and so on. Indeed, the authors never intended to provide an introduction to the whole field but only to those aspects indicated by the title. They have, however, certainly produced a useful collection of review articles which, largely, can be read independently.

F. Pacini

Aspects of lipid membranes . . .

Bilayer Lipid Membranes (BLM): Theory and Practice. By H. Ti Tien. Pp. ix + 655. (Marcel Dekker: New York, September 1974.) $39.50.

SINCE its rediscovery in 1962 by Tien and his colleagues, the bilayer lipid membrane (BLM) has been used successfully in the elucidation of previously intangible problems concerning the effects of a number of substances on natural membranes at the molecular level. The value of the isolated BLM as a membrane model lies in its simplicity of composition, structure and in its intrinsic inertness. These properties allow its accurate quantitative description as a system, which is essential for interpretation of the effects of extrinsic substances.

This book is, as the author states "a highly personal account" of research in this field. Its stated objectives are to summarise the current status of the research and to present practical methods for the formation of the BLM.

Within the book the principal successes of the past decade jostle with detailed descriptions of technique; though interesting, it will be perhaps confusing for the beginner, and of doubtful value to the experienced worker. And there are several exceptions notable by their absence: nowhere is there a mention of studies of the model unit conductance channel, possibly the most important recent development in the field, initiated by Haydon and his colleagues in Cambridge in 1970. Likewise, nowhere is there a mention of the use of step functions of current and voltage to distinguish between the effects of unstirred layers, diffusion polarisation and surface properties, studies initiated by Läuger and his colleagues in 1971.

In chapters 8–10, some 200 pages or so are devoted to electronic and photo-electric phenomena. The conditions under which some of the described experiments are carried out and the abbreviations used are not explained explicitly. As these are essential in the interpretation of the results it makes for unnecessarily difficult reading.

The content of chapter 11 ranges from detailed discussions of preparations of membrane forming chemical mixtures to a description of the apparatus for measurement of fluorescence; these are plentiful in detail but lacking in principle. The "simple experiments with BLM" included in the book belie the problems likely to be encountered in practice.

All this is not to say that there is not much useful information in this unique collection of typescript writings—there is, but its value must surely rank as a limited, expensive, and rapidly dating reference book. **Edward Lea**

. . . lipids in cancer cells

Lipids and Tumours. (*Progress in Biochemical Pharmacology*, vol. 10.) Edited by K. K. Carroll. Pp. x + 360. (Karger: Basel, London and New York, 1975.) SFr.148; £26.00; $67.50.

THE current surge of interest in the role of membranes in homeostasis and cancer ensured that a reappraisal of the role of their lipids would follow. In this area, the fluid mosaic model proposed by Singer for the structure of animal cell membranes has gained much favour and has stimulated a number of new ideas. The model considers membranes essentially as solutions of protein in a lipid environment, the fluidity of which determines the ease with which the protein components orientate in response to a number of external stimuli. Accordingly, this review on the subject of lipids and cancer comes at an opportune time and the book will attract readers from a wider spectrum of disciplines in cell biology than would have been the case a few years ago.

The contributors are well known in their fields and they have covered a comprehensive range of subjects including the glycerolipids, fatty acid metabolisms, proteolipids, lipids in cultured cells, fucolipids, control of lipid biosynthesis and the role of dietary fat in cancer. Unfortunately, the newer techniques of spin label and fluorescent probes are presumably too new to have been included.

The message that lipids in cancer cells are different from those in normal cells seems to be clear but the welter of changes described allows no coherent story to be formed. The use of rapidly growing 'laboratory' tumours carries with it many hazards of interpretation and since this material was used in most of the studies described it places severe limitations on conclusions about the relevance of the lipid changes to the tissue's behaviour as a cancer. Are the changes caused by the rapid growth of these cells? Which normal tissue provides the most appropriate control? Does the high proportion of necrotic tissue in some tumours affect the results? Lipidologists seem to have been slow to use the advantages of animal cell culture systems in which well controlled studies can be made with quiescent and growing normal cells, tumour cells, cells in different phases of the growth cycle, virus and chemically transformed cells and cells growing in medium containing delipidised serum with added lipids. In their chapter on lipids in normal and tumour cells in culture Howard and Howard point out the value of culture methods. An example of its application is the finding that the characteristic glycolipid change found in transformed cells in culture—the simplification of the carbohydrate moeity—is also found in rapidly growing normal cells. In the light of this result the degree to which this change is found in the range of Morris hepatomas can be interpreted as a function of their growth rate or, to put it another way, the time the cells spend in the phase of the cell cycle when the carbohydrate chain elongation is maximal—the quiescent or G_1 phase.

There is, however, much material in this book to prime the imagination of those working in the field of lipids and membranes. The format of the book and the layout of the chapter sections is pleasing. The texts read easily and most contributors have managed to avoid peppering them with references. It would have been helpful if the closing date for the literature survey for each chapter had been given and also if a short addendum with 'hot news' had been added to each section as late as possible before publication. The staggering price of this book (£26.00; $67.50) will ensure that it will not find its way into many private libraries. **I. A. Macpherson**

An Egyptian bas relief dating from 2540 BC showing cabinet makers at work (Mastaba of Tiye, Saqqara). The figure at the right is using a bow drill, perhaps the earliest forerunner of modern machine tools. From *Simple Working Models of Historic Machines.* Paper edition. By Aubrey F. Burstall. Pp. 79. (MIT Press: Cambridge, Massachusetts, and London, 1975.) n.p.

The Wild Canids: Their Systematics, Behavioral Ecology and Evolution. Edited by M. W. Fox. Pp. xvi+508. (Van Nostrand Reinhold: New York and London, March 1975.) £10.60.

UNTIL very recently no comprehensive review of the wild members of the dog family had been published since Mivart wrote his *Monograph of the Canidae* in 1890. Now two books on the subject have become available. The first, Lois E. Bueler's *Wild Dogs of the World* (Collins, London, 1974), is an excellent popular book; it is well complemented by the second, a more technical work entitled *The Wild Canids.* This latter volume is based on behavioural studies within a framework of five sections: taxonomy, behaviour, genetics, ecology, and the evolution of behaviour. Much of the work has been published before but this does not detract from the value of the book as a review.

The ethology and ecology of the northern races of the wolf, *Canis lupus*, have been studied in detail over the past decade and are well reviewed again here; and a new approach is used in a chapter on the Eskimo hunter's view of the wolf. There is still, however, a lack of any information on social structures in the Indian and Arabian races of the wolf and, as it is probable that these were the main progenitors of the domestic dog and the dingo, it would be most interesting to learn more about their social organisation (touched on by Lorenz in the foreword). Corbett and Newsome show that the dingo is not highly social and is a solitary hunter; whether this type of behaviour evolved since the dingo became feral in Australia, or whether it was inherent in their ancestors, provides a fascinating enigma to add to Macintosh's chapter on the origin of that animal.

Observations on the behaviour of the coyote, *Canis latrans*, and on some of the less well known canids, for example, the South American foxes and the Indian dhole, *Cuon alpinus*, are disappointingly slight. Lawrence and Bossert produce interesting evidence in support of hybridisation in the North American species of *Canis.* This work exemplifies an interesting aspect of canid behaviour that emerges in several parts of the book. This is the ability of certain species to have a close interaction with man (and with the domestic dog, with which the wolf, coyote, jackal, and dingo will all interbreed). Some species within the genera *Canis*, *Vulpes*, and *Dusicyon* are so flexible in their behaviour and ecological requirements that they can coexist with man and even take advantage of human disturbance to the environment.

The Wild Canids will be of great value to all students of animal behaviour. It has been well edited and the bibliography is comprehensive. As Michael Fox says in his conclusion, one of the aims of the book is to reduce man's alienation from nature. It should help.

Juliet Clutton-Brock

Dogs, frogs and fish

Xenopus: The South African Clawed Frog. By E. M. Deuchar. Pp. x+246. (Wiley-Interscience: London and New York, February 1975.) £10.25.

A GLANCE at the list of references within this volume will indicate to the reader how rapidly *Xenopus* has risen in popularity with research workers in the field of developmental biology.

Xenopus laevis is easy to keep because it is aquatic and thus not averse to life in aquaria, even when it is kept at temperatures much lower than those normally encountered in its African habitat. The male and female can conveniently be induced to mate by the injection of appropriate gonadotrophins which are used for human medication. Dr Deuchar suggests that the sperm may need to swim faster than the sperm of other species because the male and female cloacae are not as close as they are in other anurans during amplexus. But, while collecting freshly laid eggs, I have noticed that the male partner bends his back to bring the cloacae very close when the female is actively laying eggs, but not at other times.

Most research workers concentrate on studies of embryos and tadpoles that need not be as accurately timed as mine, but staging is all important and for this they find, as Dr Deuchar says, that the *Normal Table of* Xenopus laevis *Daudin* is invaluable. Even a group of tadpoles that have been reared together will display differences in their rates of development, though they are smaller than the differences between groups reared at different temperatures or on diverse food regimes. It is thus rather meaningless in research to refer to the ages of tadpoles alone instead of to stages which can be quite precisely used by all investigators.

Dr Deuchar has stressed the relative speed of *Xenopus* development which may be a pleasure to some but a disadvantage to others. The beautiful grace and transparency of the *Xenopus* tadpole is a delight to all.

She has read a great deal (more than 500 references) in order to produce such a comprehensive survey of the taxonomy, anatomy, physiology and development of *Xenopus.* Chapters devoted to the last of these subjects take up half of the book and provide an admirable review of present day research into many aspects of the pre-frog life of *Xenopus l. laevis.*

This book should be made available to all who work in this field. **Louie Hamilton**

The Early Life History of Fish. (The Proceedings of an International Symposium at Dunstaffnage Marine Research Laboratory, Oban, 1973.) Edited by J. H. S. Blaxter. Pp. x+765. (Springer: Berlin and New York, 1974.) DM98; $40.00.

THIS volume contains the collected contributions to a symposium on the early life history of fish held at the Dunstaffnage Marine Research Laboratory at Oban, Scotland, in 1973. Fifty four papers are published in their entirety, with two more in abstract form. The volume (like the symposium) is divided into seven categories (population studies, distribution, feeding and metabolism, physiological ecology, developmental events, behaviour, and taxonomy).

Most of the contributions fall under the heading of population studies, with discussions of egg and larval abundance in relation to fish population dynamics greatly outnumbering the other papers. High mortalities during early life have a potent effect on the recruitment of a year class to a fishery and several contributions are concerned with the causes of this: the interactions of starvation, predation, physical damage, loss to adverse current systems, and imperfections in development, are all discussed. Clearly, the causes are varied and rarely stem from any one factor.

The section on physiological ecology contains several valuable papers on environmental topics. The effects of cadmium on the development and survival of herring eggs, and the effects of thermal shock on larvae entrained in electricity generating station cooling systems, are two papers of particular relevance to fisheries in industrial regions. Another contribution discusses the effects of H_2S on the development and survival of larvae in freshwater fishes.

Under the heading 'Rearing', six papers cover techniques related to aquaculture. Variation in temperature and in photoperiod, hormone injection, selective hybridisation, and genetic manipulation all have an obvious relevance to rearing fish for food and are considered in various contributions.

These wide-ranging topics are typical of the broad spectrum of research into fish eggs and larvae that was presented at the symposium. The editing of this volume (and the organisation of the meeting) provides a lasting testimony to J. H. S. Blaxter's enthusiasm for this specialised field. **Alwyne Wheeler**

obituary

Maurice Freedman, a leading social anthropologist, died on July 14 at the age of 54.

Professor Freedman followed a distinguished career in London by becoming head of the Institute of Social Anthropology at Oxford and a Fellow of All Souls and at the time of his death was Professor of Social Anthropology at Oxford. He had entered the department of anthropology of the London School of Economics in 1946, his first work involving studies of racial and cultural relations in Malaya. Appointed to a lectureship in anthropology at the LSE in 1950, he became reader in anthropology in the University of London in 1957, and was elected to a personal chair of anthropology in 1965—moving in 1968 to an established post in this subject. But his main interests lay in the field of overseas Chinese society. In 1949–50 he spent nearly two years of field research among the Hokkien-speaking Chinese of Singapore, publishing as a result *Chinese Family and Marriage in Singapore* in 1957. For the next 25 years, he was to build on this framework to form a deep understanding of basic Chinese concepts and institutions. He clarified the structure of lineage and kinship in south-eastern Chinese society; the nature of ancestor worship; and the significance of geomancy—the art of relating a site, especially for burial, to the good fortune of men. Professor Freedman was also deeply concerned about the position of Jews in the social life of Britain.

Carl O. Sauer, who was emeritus professor of geography at the University of California at Berkeley, has died at the age of 85.

Sauer was for more than 30 years chairman of the geography department at Berkeley, retiring in 1957 as emeritus professor. He did much to establish Berkeley as a school of "cultural geography". He established the department's doctoral programme, and became a leading and widely published authority on desert studies, tropical areas, the human geography of Indian populations, and agriculture and native crops of the New World. He maintained that all geography is essentially historical. Written documents, archeological evidence, plant data, soil profiles—all provided him with clues for unravelling problems of the human global environment. His many awards included the Victoria Medal from the Royal Geographical Society, which he received recently, the Vega Medal from the Swedish Society for Anthropology and Geography in 1957, and the Charles P. Daly Medal from the American Geographical Society in 1940.

announcements

Awards

J. Bingham has been awarded the **Mullard Award** by the Royal Society for his contribution to agricultural productivity by breeding a series of winter wheat varieties.

The Potato Marketing Board has awarded a **James E. Rennie Award** to **D. N. Crowe** and **J. A. H. Taylor.**

Appointments

F. G. T. Holliday has been appointed a member of the Oil Development Council for Scotland.

The Nature Conservancy Council announce that **F. B. O'Connor** has been appointed deputy director.

International meetings

September 3–5, **Child language,** London (The Information Officer, School of Oriental and African Studies, Senate House, Malet Street, London WC1E 7HO, UK).

Person to Person

Bibliography on reconstruction from projections. Listings needed in computerised topography, radio astronomy map synthesis, multi-dimensional profile inversion, 3D electron microscopy, zeugmatography, inversion of Radon transform, and so on, all problems in reconstructing 2D and 3D images from data obtained by projection or line integration. To be published. (Richard Gordon, Image Processing Unit, National Cancer Institute, NIH 36/4D28, Bethesda, Maryland 20014).

There will be no charge for this service. Send items (not more than 60 words) to Holly Connell at the London office The section will include exchanges of accommodation, personal announcements and scientific queries. We reserve the right to decline material submitted. No commercial transactions.

September 9–10, **Morphology and biology of living and fossil reptiles,** London (The Executive Secretary,

The Linnean Society, Burlington House, Piccadilly, London W1V OLO, UK).

October 1–10, **High energy radiation dosimetry and protection,** Sicily, Italy (Dr A. Rindi, Building 72, Room 122, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720).

October 6–8, **Fertility and sterility,** Madrid, Spain (Professor E. V. Dominguez, Feneral Pardines 82-c-70 DCHA, Madrid, Spain).

Miscellaneous

Neuberger report. The British Nutrition Foundation has decided to institute an essay competition on 'Nutrition is an emerging subject; discuss in relation to the Neuberger report'. The competition (essay length up to 2,500 words) is open to young scientists and medical students under 25 and prizes of £100, £75 and £50 will be awarded. Entries (before December 31, 1975) to: Mr P. M. Victory, Secretary, British Nutrition Foundation, Alembic House, 93 Albert Embankment, London SE1 7TY, UK.

How companies benefit from the CERN connection

EVERYONE knows that the non-stick saucepan was a technological spin-off from the Apollo space programme, and everyone knows that you have to think quite hard if pressed to name a second product with which the ungrateful world of consumers has been accidentally endowed by high technology. But space exploration is not without its benefits of a sort. The vast technology-based industries that have sprung up to serve space and for that matter, military needs, can be seen as (if nothing else) a means of keeping large numbers of people in employment. Inevitably, however, one asks whether the development, say, of better space propulsion units by industry, when there is only one customer, serves any broader purpose, and almost equally inevitably one answers that it does not. The rapid break-up of many space-and-defence-based industries when the big client loses interest or runs out of money is testimony to the problems of industry capitalising on the push of technical demand when that push is on too narrow a front.

Not every major new project based on science and technology comes from aerospace, however. It is fascinating to ask whether anything rubs off on an industrial company which performs contract work in the construction of nuclear reactors, radiotelescopes, deep-sea drilling equipment or whatever: and a fascinating answer is given in a recent and highly readable report published by CERN (*A Study of Economic Utility resulting from CERN contracts*, H. Schmied, Report no. CERN 75–5). The diligent Dr Schmied conducted 110 interviews with CERN staff and ultimately identified 127 companies throughout Europe where there was a possibility that dealings with CERN had created 'utility' for the company beyond the contract itself. The utility could spring either from increases in added value in subsequent sales of products elsewhere or from cost savings in subsequent operations. Increases in added value might arise from the sales of new products originally developed for CERN, increased sales of existing products owing to the CERN connection, improvement of a product to meet a tough specification and subsequent sales of the improved product, or by other means. Cost savings could arise from the indirect use of CERN expertise in research, development and production, from savings on capital investment when CERN contracts had already partially covered costs and from savings in marketing from the use of CERN as a reference.

Companies were asked to make their estimates (using guidelines provided) of the utility of having worked on contract. Obviously, and Dr Schmied acknowledges as much, there is no foolproof way of identifying utility; some companies find it difficult to accept that anyone beyond their own four walls could possibly have contributed anything of significance, others might think a high value for utility would please CERN and perhaps land more contracts whilst yet others might think a high estimate could cause CERN to adopt a more hawkish attitude in its future letting of contracts. And the measurement of utility is itself bound to be a fairly hit-and-miss affair. Nonetheless there are some very striking conclusions.

If all 127 companies are taken together, their sales to CERN amounted to 394 million Swiss francs (MSF), but the utility generated came to 1,665 MSF: the CERN connection had borne fruit fourfold. There are obvious gradations. Manufacturers of standard electrical equipment such as cables have little to gain from working for CERN. At the other extreme manufacturers of computers and precision engineering reported an average of 17 and 32 respectively for the utility/sales ratio. And companies in the UK, Norway, Sweden and Denmark have done particularly well, undoubtedly because the CERN contract has opened up new geographical market horizons.

This study does not say that the world is economically a better place because of CERN, or that economic activity is created which would not have existed otherwise; it is entirely conceivable that a study of companies unfavoured by such contracts could identify losses to counteract the gains of the favoured companies. What it does say is that individual companies demonstrably profit in many ways from association with large-scale high technology. And this is particularly interesting in CERN's case, because none of the companies seems to have been exclusively dependent on CERN for its turnover, and a very large part of the utility achieved has necessarily to be outside the narrow realms of particle accelerators.

It would be wrong to take these figures as a good measure of the extent of technology transfer possible through major technological enterprises, as some of the utility has been gained by the simple process of stamping "as supplied to CERN" on the glossy brochures. But industrialists could well take note of the financial advantages of working with high technology. And governments involved in big projects should ponder whether the acquisition of large numbers of their own technical staff for specific jobs is the best way to take the broadest possible national advantage of the investment.

Net profits

C. E. Purdom and A. Preston of the Ministry of Agriculture, Fisheries and Food, Fisheries Laboratory, Lowestoft, write about the prospects for fish farming in the UK.

THE annual world production of farmed fish is approximately 4×10^6 tons which represents about 15% of supplies of fish for direct human consumption. In the UK, production at present is about 1,500 tons a year, or less than 0.2% of consumption. Precise statistics are not available, but a considerable proportion, perhaps half, of the production is for conservation or sport purposes.

The fish farming industry in the UK comprises a variety of small, mostly privately owned, farms and a few large companies. About 100 private farms exist, each producing between 5 and 200 tons per year with by far the greater proportion at the low end of the scale; in addition, about 30 farms under the control of Regional Water Authorities produce fish for re-stocking purposes. Small scale fish farming has a history going back about 100 years. It is traditionally associated with the production of trout for sport purposes, although current expansion also includes food production. Involvement by large companies is very recent and is still in an early developmental phase, with production primarily for food supplies. Output is still low and geared to the supply of luxury food in the form of trout and salmon, with research emphasis on the latter. So far there is little input into the mass retail industry and marketing methods remain traditional.

Apart from a very minor effort with cyprinid fish for re-stocking, fish farming in the UK is at present restricted to salmonids. The culture in fresh water of native brown trout (*Salmo trutta*) and North American rainbow trout (*S. gairdnerii*) is the dominant element, with sea-water culture of salmon (*S. salar*) restricted to large companies with production levels of only a few hundred tons per year at present.

Marine fish farming research in the UK was initiated by the Ministry of Agriculture, Fisheries and Food (MAFF) about 15 years ago but its origins go back much further than this and probably to the turn of the century when hatcheries were established for plaice (*Pleuronectes platessa*) and cod (*Gadus morhua*) to produce fry for liberation into the sea for conservation purposes. This ineffective measure was discontinued by about 1920, but the concept of farming marine fish seems to have grown out of the experience gained in the production and hatching of eggs of the plaice. Thus initial research was hatchery orientated and directed towards plaice farming. Hatchery techniques were successfully developed and large scale production of juvenile plaice became feasible by 1966. At this time the White Fish Authority (WFA) entered the field to develop the rearing techniques towards commercially viable levels and, especially, to study the growing-on phase from juvenile stages to marketable size. It was in this area that economic problems with plaice became apparent; their food bill exceeded the first-sale value. Attention was therefore switched to sole (*Solea solea*) and turbot (*Scophthalmus maximus*) for which better economic returns might arise. Sole has proved more intractable than plaice during on-growing but turbot has been shown to be very amenable during this part of its life cycle. A basic requirement in intensive fish farming is that food is taken rapidly and not wasted or allowed to decompose and pollute the environment. Turbot fulfil this requirement but sole, with present rearing techniques, do not, being slow, cautious feeders. Progress in turbot farming has been restricted, however, by serious difficulties encountered in the production of juvenile fish and present research on farming techniques depends, in part, on the collection of juveniles from wild stocks. For conservation reasons this sets a restrictive limit on possible total commercial production for this species of about 100 tonnes a year. The earliest attempt to rear turbot was made by Anthony in 1910 but the first hatchery reared juveniles were not produced until 1972 and then only in very small numbers. Improvements have been made in succeeding years, during which the development of turbot hatchery techniques has had considerable priority in government laboratories, but the methods still fall short of the level of achievement necessary for commercial exploitation, although one major food company is exploring the hatchery production of turbot with the assistance of MAFF.

Fish farming in the UK has grown slowly in the freshwater and marine salmonid field under commercial patronage, although some acceleration has occurred over the past 2 or 3 years; on the other hand, government research, largely under the auspices of MAFF, but with the later involvement of the WFA and the Natural Environmental Research Council (NERC), has been preoccupied with the development of methods for rearing marine flatfish. So far no major research work on salmonid farming has been undertaken by government agencies, and commercial interest in marine flatfish has been limited, largely to a joint project between the WFA and one major food company. This dichotomy is in marked contrast to the situation in other parts of the world, such as North America, Japan and Norway, where government and private concerns are mutually and extensively involved in a wide variety of fish farming projects.

With little prospect of increasing the direct yield from the sea of species of prime fish, and many indications that the reverse might occur, it has become necessary to examine the potential for an expansion in fish farming in the UK. In this context a review was commissioned with the object of drawing up a MAFF programme of research and development into marine and freshwater fish cultivation and assessing the facilities required to carry it out. This review, which took into account the existing scope of fish farming activities, formed the view that a viable industry needed to be broadly based and to cater for both the small farmer and major commercial interests. Some legislative adjustments may be necessary in this context but the primary objective should be to provide, through a well conceived research and development programme, a sound scientific basis for the expansion of fish farming into a broadly based national industry.

The question of which species of fish to cultivate is crucial to the development of a research and development programme of relevance to an expanding industry. Among the salmonids, rainbow trout and Atlantic salmon are obvious choices in view of their present commercial status; brown trout and sea trout may also be of value within the context of marine culture of salmonids. Among the marine flatfish, turbot alone seems to have potential at this time, although sole would have definite economic advantages if the feeding problems experienced in the growing-on phase could be solved. Other freshwater and marine species do not warrant attention at present but should be subject to periodic review in terms of their potential for cultivation

The research and development needs differ between species. The principal subjects areas for investigation are hatchery studies, nutritional requirements, water management studies, facility development, genetic manipulation and disease control. Of these, hatchery techniques, nutrition and disease control are well established for salmonids after more than a century of development. There is still room for much improvement, particularly at the nutritional level, and disease control and treatment require continuous study. The major constraint to a large scale expansion in trout farming in fresh water, however, lies in the nature of the water requirements. Rainbow

trout farms use about 30,000 gallons of high quality fresh water for each pound of fish produced and the availability of sites for expansion is very limited. Two methods for overcoming this constraint are the use of recycling systems and the culture of trout in sea water. The former can be regarded as a technological development based on present practices, whereas sea-water culture is a more radical change with many facets requiring investigation. Although no major research work on salmonid farming has been undertaken by MAFF or the Department of Agriculture and Fisheries for Scotland some facilities are available and plans for additional facilities are in hand: in these, special attention will be directed towards nutritional studies, cage systems in the sea, recirculation systems for fresh and salt water, and genetic studies aimed at producing improved strains. Disease research and monitoring is already covered both in England and Wales and in Scotland.

Flatfish farming research has not yet established a sufficiently reliable technology for full commercial development. The first priority for further work lies with turbot and especially with the need to overcome the problems of hatchery production of juveniles.

One major area of work of fundamental importance to any form of fish farming is the development of a genetic approach to produce improved strains, to avoid inbreeding depression and to conserve genetic uniformity and hence performance in domesticated lines of fish. A further useful field with genetic implications is the development of all-year-round spawning stocks and the control of sex itself. Genetic manipulation at the hatchery level is at present studied within the MAFF programme, but no facilities exist for extending this to the growing-on phase or for holding stocks of domesticated fish. For marine flatfish, the creation of domesticated lines has not yet begun. Similarly, Atlantic salmon stocks are basically of wild origin but rainbow trout exist in a wide variety of selected lines, particularly in North America. Although a proper evaluation of their merits, and the possibility of heterosis in F1 hybrids, seems not to have been attempted, this is an area of study which could have an immediate beneficial effect on farming practices in the UK.

In conclusion, there is an established and expanding industry for salmonid farming, principally in fresh water, and a growing interest on the part of large companies in marine flatfish culture. In view of the likely diminution of supplies of prime quality fish like sole and turbot from the sea it is desirable to encourage the continued expansion and development of this area of food production, on a broad basis and at all levels of commercial involvement, though this should not be construed as implying any substantial contribution to offset a short-fall in basic supplies. To this end the programmes of research and development in the fields of hatchery production, environmental control, nutritional innovation, genetic manipulation and disease identification and control within the species at present exploited or showing potential for exploitation require careful integration. The list of species at present includes rainbow trout, salmon and turbot with secondary consideration for brown trout, sea trout and sole. □

Funk therapy

THOMAS H. JUKES

The term "vitamin(e)" was coined by Casimir Funk in 1912. He was good at promoting its use, but he could never have foreseen the mind-expanding effect of this new word in the 1970s. Vitamins are coenzymes and other metabolic catalysts whose pathways of biochemical synthesis have been lost as a result of mutations during the evolution of animals Identification and synthesis of vitamins led to spectacular successes in preventive medicine; these became well known, and, as one result, popping vitamin pills is part of the American way of life, just like colour television in the family room. The idea is not so much that diets are deficient, but why not take vitamins "just to be safe"? Indeed, except for a half-hearted attempt some years ago by the US Food and Drug Administration (FDA) to throw cold water on it, the habit had few opponents and it was certainly better than smoking cigarettes.

There is, however, a basic pharmacological delusion of the layman that if a small dose of a medicine is good, a large dose must be even better, especially if it is not poisonous Many physicians have treated patients whose symptoms exceeded in severity their physical diagnosis, by prescribing "a good rest with lots of vitamins". This procedure is known as "placebo therapy"; the administration of a harmless nostrum for soothing the nerves of the apprehensive, and for convincing those who are open to suggestion that they are receiving "medical treatment". Vitamins can be sold without prescription, and the medical profession, by fostering the use of vitamin pills, made an opening for nonphysicians to practice medicine without a licence. The "health-food" industry responded, and its sales outlets stock pills containing vitamins and alleged vitamins. If health food is so "nutritious", why does it need supplementation with large doses of vitamins and minerals? The answer given in the health food literature is that such dosage will produce super-health, an effect not measurable in human beings or laboratory animals.

Vitamins go through periods of fashion. Thiamine was in the spotlight at one time and, more recently, ascorbic acid became the darling of the headlines. Vitamin B_{12} is popular, probably because it is often injected and is red. Vitamin B_6 is all the rage now, and vitamin E is a hardy perennial because it was lucky enough to be discovered by the fact that its deficiency causes infertility in rats. The same is true of vitamin A, of course, but E has ever since worn the halo of Aphrodite, and has collected many unearned credits.

"Megavitamin therapy" has its devotees, and publicity is currently given to "orthomolecular psychiatry", defined as the "achievement and preservation of good mental health by the provision of the optimum molecular environment for the mind, especially the optimum concentrations of substances normally present in the human body, such as the vitamins". Whether or not this actually works we shall probably never know, because the author (Linus Pauling) also says "the principles of medical ethics prevent orthomolecular psychiatrists from withholding from half of their patients a treatment that they consider to be valuable. Controlled tests can be carried out only by skeptics".

Efforts of the FDA to regulate megavitamin promotion, however, were set back by a court decision and by the passage last year of a bill in the US Senate that specifically prevents the FDA from classifying high-potency vitamin preparations as drugs.

international news

CAB move with the times

from a correspondent

THE Review Conference of the Commonwealth Agricultural Bureaux, which has just ended in London, could be a landmark in the history of the organisation. The CAB was set up in 1929 to provide an administrative centre for the group of bureaux, each of which acted as a clearing house for information in a different branch of agricultural research. Since then the group has expanded, and it now comprises nine bureaux, which function as originally intended, and four institutes, which have somewhat different functions. Although only an 'organisation', in the loosest sense, the bureaux have maintained, and justified, their reputation for providing the world's best and most comprehensive sources of information in the fields they cover. Nonetheless, and perhaps because of their very success, the bureaux have in recent years appeared to be somewhat conservative, even at times complacent, as regards their own functions and performance.

The CAB has three rather distinctive functions:
● A global documentation service covering virtually the whole field of agricultural and allied research in a series of abstracting journals.
● A taxonomic and identification service, provided by the three institutes dealing respectively with entomology, mycology and helminthology.
● An operational service provided by the Commonwealth Institute for Biological Control.

From these springs a fourth function, the operation of a 'mutual assistance' agency that provides Commonwealth countries with very much more in the way of information and services than they could obtain for themselves at anything like the same cost and effort.

The annual budget of the CAB totals slightly more than £3 million, of which about half comes from the subscriptions of member countries, and another £1·5 million from the sale of bureaux publications. Only about 30% of the output goes to Commonwealth countries, and because these can buy their information at a reduced rate, no less than 85% of the income from these services comes from outside the Commonwealth. The major 'foreign' clients are the United States, Japan, and one or two European countries such as the Netherlands and Germany. The question of the cost-effectiveness

of the bureaux operations was a principal subject of discussion.

Taking first the documentation services, it was felt that these could and should be more self-supporting than at present, particularly in view of the increasing cost of production and materials. So the CAB has been taking a hard look at some other internationally used abstracting services, such as International Retrievals (IRS), who are believed to make a profit, and INSPEC, which claims to break even, more or less. One result was a resolution that the bureaux should "seek to associate themselves with non-Commonwealth partners, national and international, as consortia". This is already done with Food Science and Technology Abstracts, providing an international food industry information service in consortium with the German company IDW.

One obvious development ought to be collaboration with the AGRIS system now being sponsored by the FAO from Rome. The CAB sees itself as complementary to, certainly not competing with, AGRIS, but the strong anti-national prejudice that at present surrounds all UN-oriented operations would probably make close collaboration impossible at present.

On the technical side, the CAB intends to make much more use of modern computerised production methods, including increased use of machine-readable material, which the bureaux have been noticeably slow to adopt.

The situation as regards the work of the three institutes is very different from that of the bureaux, Their services have always been given free, only the Commonwealth Mycological Institute having a small income from the sale of cultures of fungi. A small step is now being taken, whereby a charge of £10 an item will be levied for all identifications done for non-contributing governments or com-

mercial clients The case of the Commonwealth Institute for Biological Control (CIBC) is different again. A suggestion that its headquarters might be moved from Trinidad back to the UK was resisted by the representatives of the less developed Commonwealth countries. But there was general agreement that the institute should in future concentrate on fewer, bigger projects rather than providing a 'fire service' to deal *ad hoc* with any problem that might arise within its terms of reference.

In line with efforts to streamline the services of the CAB, the CIBC's outstations will also be reduced to four in number, strategically situated in accordance with a new, more closely regional policy. They will be sited in Trinidad, in Delemont, Switzerland (for forest pests mainly in North America) in Kumasi, Ghana and in Bangalore. Smaller outstations will be run down and, in future, work not done at the four main centres will be on an *ad hoc* project basis.

Finally, there are to be changes and expansion in the central management of the CAB. The present small secretariat, with only four senior officers, will be reinforced by the appointment of a marketing manager (although in deference to the inhibitions of some of the older bureaux staff, his official brief will be "circulation and services") two editorial managers and a scientific coordinator. More problematic will be the question of regrading and even redundancy in certain staff categories. This may well have to wait until some of the more conservative among the present Directors of Bureaux have retired. The fact is that these have hitherto traditionally been senior scientists with great experience in the discipline for which their bureaux are responsible. But under modern conditions, what is probably needed is a group of highly expert information scientists, ready to take advantage of every new aid to quicker, more efficient production of the Abstracts, more prepared to enter into agreements with other organisations and perhaps less inclined to feel that if the CAB doesn't do it, it isn't worth doing. Many of the changes that must come will have to be made anyway, and in most instances the sooner the bureaux can meet changing circumstances half way. the better for everyone. ☐

Dr Gerald Stanhill has recently come to the conclusion that his Biblical and post-Biblical ancestors showed remarkable powers of scientific observation and analysis. Dr Stanhill, who heads the Division of Agricultural Meteorology in Israel's Agricultural Research Organisation, points out that the traditional Jewish prayers for rain, codified almost two thousand years ago, are most frequent and most intensive at precisely those times of year when additional moisture will do most to raise wheat yields in the Holy Land.

Scientific studies carried out over the past 20 years indicate that above average rainfall in the early winter immediately after sowing increases yields, as does bountiful precipitation after the wheat has flowered. In contrast, rainfall at the beginning of the season (before sowing has taken place) and in midwinter (between tillering and booting) depresses yields. And, indeed, prayers for rain become most frequent and high pitched just after sowing (which took place in Biblical times, and takes place now, in November). If rain does not fall in spite of the prayers, a series of fasts is prescribed: at first only for the sages, then for the entire public from dawn to dusk and finally, if the dry spell continues, 24 hour fasts are to take place twice each week.

Both religious practices and archaeological digs indicate that Jewish farmers of the Biblical period were growing wheat primarily in the southern part of the country (where rainfall is both scarce and uncertain) or in the hilly regions (where the soil is thin and does not absorb a great deal of moisture at one time). Today's most prosperous agricultural areas, where rain can be counted upon, were not cultivated for the most part by the Israelites. Interior lowlands, like the Huleh and Jezre'el Valleys, were soggy swamps, and remained so until they were drained a few decades ago.

● While the draining of the swamps increased the supply of fertile farmland and contributed to the elimination of malaria, it has not always proved an unmixed blessing. As a result of the elimination of the Huleh Swamp, for example, the Sea of Galilee is facing a pollution threat.

The peat of the former swampland is heavily impregnated with nitrogen, and when water flows through the Huleh and into the Sea of Galilee, some 15 km to the south, it carries with it large quantities of nitrogen. This influx, many scientists believe, increases the growth of aquatic vegetation in the water, thus contributing to the pollution of Israel's central fresh water reservoir.

A number of researchers have suggested that the problem be solved by planting crops in the Huleh Valley which would consume the excess nitrogen. Dr Avraham Hartzook of the Agricultural Research Organisation, for example, has in mind a plant known as Kenaf (*Habiscus cannabinnus*), which would not only deal with the nitrogen question, but might also reduce the country's dependence

Letter from Israel

from Nechemia Meyers

on increasingly expensive and scarce wood pulp for the production of paper.

Some time ago Hartzook helped to introduce Kenaf to Tazania, where he spent two years as an Israeli agricultural expert. Now a successful cash crop in that country, its fibres primarily serve as a substitute for jute, but they have also been used, albeit on a small scale, as a substitute for wood pulp by the paper industry. Dr Hartzook sees a considerable potential for Kenaf in Israel, which has little wood of its own and might therefore be ready to exploit the plant earlier than other, more richly endowed countries.

● Pollution problems in the Gulf of Eilat are, if anything, more serious than those in the Sea of Galilee according to Professor Ralph Mitchell, now on a working visit to the Weizmann Institute from Harvard University.

Mitchell, who has done extensive studies of the Gulf under the sponsorship of the United States Office of Naval Research, says that if pollution, particularly oil seepage, continues unchecked, the beautiful coral reefs of the area will be eaten away in 25 years.

Mitchell has also done studies on how oil affects the biological behaviour of inter-tidal snails. Some stop bunching together (to keep from being washed away), and others simply stop eating.

Oil likewise affects the ability of bacteria to detect their food, thus interfering with their vital role as marine self-purifying agents. Following the discovery that motile marine bacteria are chemotactic to many of their food sources, Mitchell demonstrated that sub-lethal concentrations of oil inhibit this chemotactic process so that the rate of substrate decay is retarded.

Mitchell is concerned not only by oil seepage, but also by the possibility of a major oil spill from one of the big tankers that wend their way to Eilat.

● Israel's mathematics teachers have established their own emergency group to fight a Ministry of Education ruling that mathematics will no longer be a compulsory matriculation subject for secondary school students.

Cutting the teaching of mathematics, they argue with great vehemence, would undermine secondary schools and hamstring universities, forcing the latter to disregard matriculation certificates and devise a battery of entrance examinations. Even worse, according to the teachers, the Army would find itself short of men with the kind of mathematical background required to handle increasingly sophisticated modern weapons.

The controversy can only be understood against the background of Israel's educational system, which differs considerably, for example, from that in the UK. Students are not given an 11-plus examination, and when they finish elementary school, a great majority continue on to a secondary modern school.

The last two years of secondary school do bring a measure of specialisation similar to that at A-level in Britain. But, even so, students concentrating on mathematics and science have thus far had to pass courses in history and literature, while those interested in the humanities have had to prove their abilities in science and mathematics in order to obtain a matriculation certificate.

Though there had long been complaints about the burden these requirements imposed on students, demands for a change only came to a head several years ago when the Education Ministry revealed that 40% of the humanities students were failing matriculation mathematics.

As an interim measure, the examination was made easier, thus allowing a much larger percentage of examinees to slip under the wire, but this only disguised the problem without solving it. So the ministry decided on more drastic action: students in the last two years of secondary school, it announced, could choose to study maths at various levels (the higher the level, the more points towards a matriculation certificate), or not study maths at all.

Wheatstone's wheezes on show

by John Hall

IT comes hard to be told that Sir Charles Wheatstone actually didn't invent the bridge of the same name, but such was the range of that scientist's inventiveness and imagination that one feels he must, on balance, be forgiven what amounts to a fairly minor omission. Bridges apart, he made fundamental contributions to the study of electrical measurement in the 1830s and 1840s, and in the process he happened across principles of transmitting electrical impulses which formed the basis of modern telecommunications systems. As if that were not enough, he spent his twenties experimenting with musical instruments, and managed as the *pièce de resistance* of this period an invention which was to become the fisherman's friend. Letters patent granted by George IV in 1829 described his creation as "a certain improvement or improvements in the construction of wind instruments"; it was the concertina, successor to earlier brainchildren like the 32-key Wheatstone symphonium (pocket-sized and powered by breath). and precursor of mighty wheezers like the 81-key Wheatstone duet, with the range of a piano. The inventor's nail fiddle, a poor ·relative of the African thumb piano, made of nails to be scraped with a violin bow, was less of a raging success.

But Wheatstone of Wheatstone's Music Shop, Islington. clearly had a novel turn of mind, as the scientific community discovered when he was appointed Professor of Physics at King's College, London. His interest in the transmission of sound vibrations led him to experiment with the transmission of electrical impulses, and to measure their speeds along wires. His success in sending information by this means led to the needle telegraph on which the developing railway system came to depend for its signalling, the ABC telegraph, an automatic Morse transmitter, a punched-tape Morse transmitter and Wheatstone's printing telegraph, which in operation was akin to a Telex printer. For the record, Wheatstone's contribution to the measurement of voltage, current and resistance was that he produced an accurate calibrated variable resistance to replace the unreliable galvanometric instruments available at the time. Later prophets will also be sad to learn that the linear motor was invented by Wheatstone in 1842: given a larger current, it might have proved his brightest wheeze.
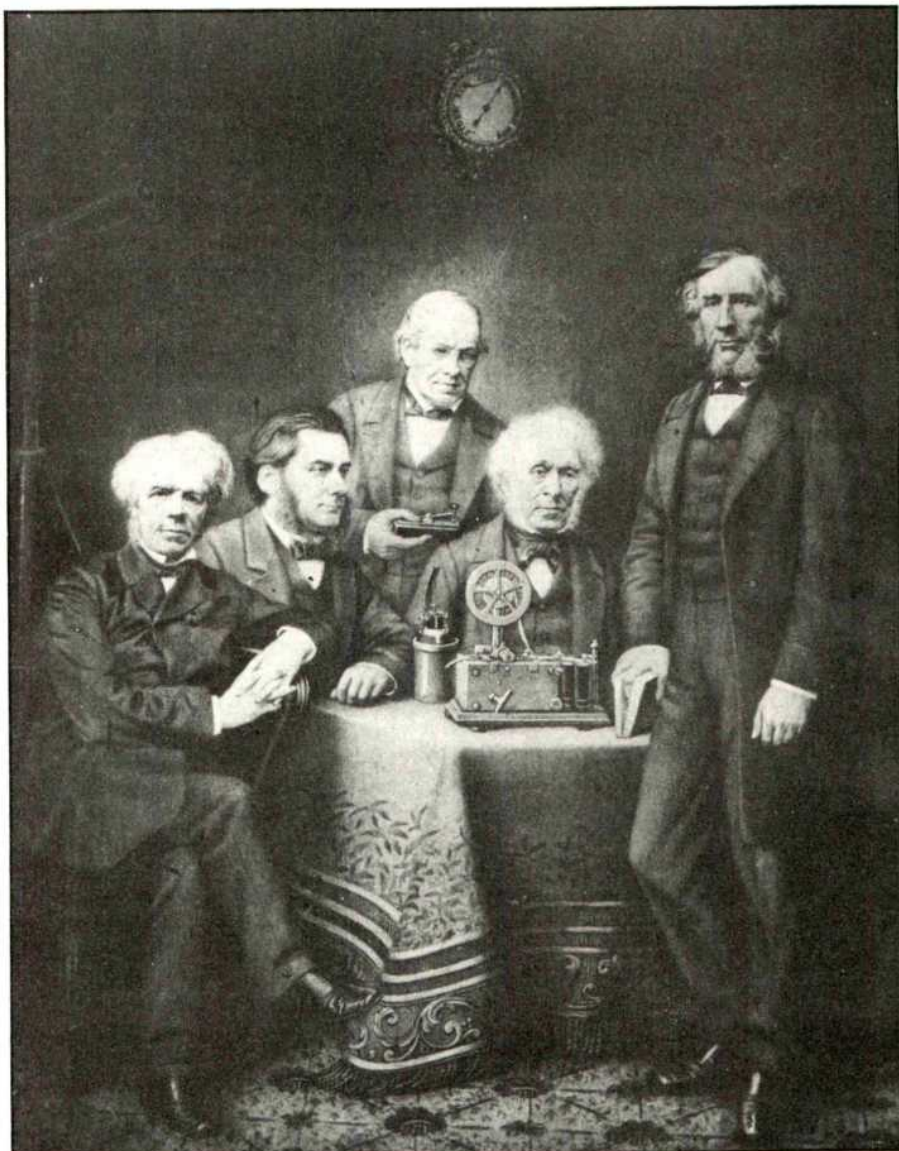
He was also responsible for the introduction of the Royal Institution's practice of locking lecturers in a room immediately before they are due to lecture; apparently, on one occasion when he was about to give a public discourse, Wheatstone's nerve went at the last minute, and he disappeared from the scene, leaving Faraday to *ad lib* the evening away.

Wheatstone is one of two eminent Victorian members of King's College to be honoured by an exhibition there this summer. The other is Sir Charles Lyell, an equally prominent and worthy man in his field of geology, but affording rather less tangible tokens of his life's work than does Wheatstone. Lyell established the main principles of geology, and amazed the learned of his day by showing that the Earth was about 4,000 million years old when you would have sworn it wasn't half that age. This scale managed to accommodate the Darwinian theory of evolution, and also gave cosmological theories a realistic time perspective, but doesn't sit conveniently in an ex-hibition display cabinet. By way of compensation there are some nice photographs of the Lyell family seat, a moderately accomplished watercolour by the man himself, and a body of evidence which testifies to the meticulous nature which the documentation of his theories must have demanded. Even as a child he collected shells and bones for his private museum, and when he wrote home to Papa it was in Latin. But he was not such a scholar that he closed his eyes to the exigencies of the world outside his books: after a page and a half of Latin, written from Midhurst Grammar School in 1814, he blurts out in the mother tongue: "If I write any more in Latin I shall miss the post."

The exhibition, at King's College, Strand, London, continues until September 10.

The picture shows the shining lights of Victorian science (left to right): Michael Faraday, Professor T. H. Huxley, Sir Charles Wheatstone, Sir David Brewster and Professor John Tyndall. □

THE British government is to draw up codes of practice for some experiments involving the genetic manipulation of microorganisms which could pose a potential hazard to workers and the general public. Mr Fred Mulley, Secretary of State for Education and Science, in answer to a parliamentary question, announced the setting up of a working party, which would

● draft a central code of practice and make recommendations for the establishment of a central advisory service for laboratories using the techniques available for such genetic manipulation, and for the provision of necessary training facilities

● consider the practical aspects of applying in appropriate cases the controls advocated by the Working Party on the Laboratory Use of Dangerous Pathogens, chaired by Lord Godber, which reported earlier this year.

Mr Mulley also asked the Research Councils and others concerned not to proceed with work already identified as involving a potentially serious hazard, pending advice from the working party.

The question of potential hazards inherent in the new techniques which allow DNA of different bacteria and viruses to be linked together and introduced into other bacteria was con-

Government codes for gene manipulation

by Eleanor Lawrence

sidered by the Working Party on the Experimental Manipulation of the Genetic Composition of Microorganisms, set up by the Advisory Board for the Research Councils and chaired by Lord Ashby. This committee, which reported in January of this year, came to the conclusion that such experiments should be permitted but only with certain safeguards.

These conditions include the use of experimental microorganisms which have been rendered relatively innocuous by specific mutations, and that certain types of experiment should be carried out under conditions of the strictest bacteriological security, in laboratories where the risk of escape to the outside or contamination of workers is minimised.

The definitive code of practice approved by the government, which the new working party should provide, will be welcome since it should end a period of delay and uncertainty which started a year ago with a call for a moratorium on a range of experiments by a committee of the National Academy of Sciences in the United States. At the same time the working party will be concerned with the practical aspects of implementing the findings of the Godber Committee which considered the use of proven pathogenic bacteria and viruses in laboratories throughout the UK. This committee drew up a list of organisms which could only be worked within conditions of strict security, which in the UK would confine such work to the few existing laboratories with suitable facilities. □

Shutting the GATE

by John Gribbin

THE World Meteorological Organisation (WMO) has been stirred into issuing a formal denial that the GARP Atlantic Tropical Experiment (GATE), conducted last year by ten nations and probably the largest and most complex international scientific experiment ever undertaken, was an experiment in hurricane control which adversely affected weather conditions in some countries.

The need for this denial stems, according to the WMO, from a statement made by "a professor of the

University of Mexico", and the WMO says that "it would indeed be regrettable if such unjustified statements were to detract from the acknowledged success of this international scientific research venture". Since the purpose of GATE was to monitor natural changes in the atmosphere and oceans, including large scale tropical disturbances, it would have been rather stupid of the investigators to attempt to alter the natural state of affairs, and most meteorologists are well aware of the need to understand natural changes in atmospheric processes before trying to induce changes by artificial means.

Perhaps the professor was confused by the inclusion of the word "experiment" in GATE's name which, al-

though in the acronymic tradition of the WMO, is perhaps a slightly misleading title for an entirely observational project. But since Mexico was one of the participating countries, it should not have been too difficult for the critic to check his facts before issuing a public statement. The lesson to be drawn, perhaps, is that research into weather and climate is such a sensitive area that bodies such as the WMO should expect to be subject to the same intensive scrutiny as agencies responsible for nuclear research, or laboratories involved in genetic engineering.

In that case, perhaps GARP Atlantic Tropical Study might have been a happier choice of name. □

correspondence

PAC report: a failure of communication

THE Indian Public Accounts Committee report "Foreign Participation or Collaboration in Research Projects in India" (discussed in a leading article on July 31) was critical of the Bombay Natural History Society, as well of the Research Unit for Genetic Control of Mosquitoes. In this letter, Dr A. N. D. Nanavati, Secretary of the Bombay Natural History Society, considers some implications of the report.

THE Public Accounts Committee (PAC) has expressed grave doubts about a number of research programmes carried out with foreign collaboration in India. Much of their alarm stems from the fact that the knowledge obtained from such researches can have applications in chemical and biological warfare (CBW) and that the administrators examined by them did not seem to be fully aware of the fact. Based on this finding the committee has formed an opinion implied, though not explicitly stated, that those concerned in these

projects were dupes, sometimes maybe even willing tools (in view of the relatively high salaries they received) in the hands of foreign organisations who wished to use India as an experimental arena for such studies.

I do not propose to go into the details or to try to refute the numerous misunderstandings and errors in the evidence on which these opinions were based. The facts of which I have personal knowledge, namely those connected with the Bombay Natural History Society (BNHS)—Migratory

Animal Pathological Survey (MAPS) Bird Migration Study, have already been stated in your columns (November 29 1974) and, I believe, adequately answer every criticism voiced against the study originally by Dr Jayaraman, and later endorsed by the committee.

I need not repeat these arguments here except to point out that Dr Salim Ali's *cri de coeur* to Dr T. Ramachandra Rao, then the Director of the Virus Research Centre (VRC), Poona, requesting assistance for the studies on ectoparasites and blood smears of ringed birds has been completely misinterpreted At the outset of the programme between the WHO and the BNHS, studies on this aspect were made by the VRC, Poona, and details of their findings were published from year to year in the Annual Reports of the VRC. When the WHO withdrew from the study, the VRC also withdrew, as it had no staff to spare for this work. Apart from occasional seasonal trips by the Institute of Natural Foci of Infectious Disease (NFID), Omsk for collection of such material (reports published in Russian), no other organisation came forward and this material remained unstudied until the MAPS organisation came on the scene. After the first year, they also requested that no more blood smears be sent as they were not in a position to study them. It was this lack of interest, and not any secret removal of data, which occasioned Dr Salim Ali's appeal to Dr Rao (who also found himself unable to help). These "orphan" blood smears are still with the society, awaiting a godfather to take an interest in them. There are several other misconceptions in the report, for example, the importance attached to Dr Theiler's suggested hypothesis on Yellow Fever epidemiology. This remains no more than a hypothesis, because it fails to explain all the observed phenomena. But these misconceptions are not of primary importance. What is important is that the PAC did not examine any scientist directly working on the projects, except Dr T. Ramachandra Rao, the officer on special duty, whose position was rather peculiar and is further discussed later. Had they examined the scientists, rather than the administrators and administrative scientists, who were only nominally concerned with the actual work on the projects, they might have revealed a completely different picture. In the case of the BNHS study, an examination of the facts will readily show that every working scientist was aware of the possible implications and repercussions, that the programme of work was formulated by the Indian Chief Investigator, and that adequate safeguards against secrecy were in force.

This brings us to the question of how far the administrator should be aware of the different facets and possibilities of any research study he is required to sanction. He must obviously rely on the opinions of his experts. Is it then incumbent on the experts concerned to appraise the administrator of all the various possibilities inherent in a proposed research study, and is such a procedure really necessary or relevant or even possible? When a project is being prepared with a particular object in mind, it is necessary to establish how far that the object will be met by the study. The expert has to advise on (a) the importance of the problem (so that it may be judged in proper perspective in the national contest), (b) the type of information which needs to be obtained to solve the problem and (c) the extent to which the proposed study can be expected to yield the required information. This is all the administrator needs to know to enable him to assess the project correctly and to judge its requirement in men and materials and its anticipated benefits relative to the many other demands which may be made on his resources. Anything else is irrelevant. Considerations like possible misuse of the knowledge gained, the safeguards necessary, and so on, must be the responsibility of the experts on the job. For example, a few years ago a study was made on the spread of cholera from village to village in West Bengal and the mechanisms by which the village wells got contaminated Every student of medicine and microbiology knows that the understanding of such process can be used either for countering the spread of cholera or for its enhancement, that is for biological warfare using cholera germs. Yet, had the administrators concerned been questioned about that project they would not have shown any greater awareness of such dangers than in the present case.

The committee's conclusions lose much of their significance because they were not, in fact, based on the evidence necessary for a correct appreciation, the evidence of the scientists concerned on the job. The only working scientist examined was Dr T. Ramachandra Rao, who made it clear that knowledge is neutral and the only safeguard against misuse of knowledge is its free publication.

The committee's criticisms and apprehensions about the detailed programme and sequence of studies made by the Research Unit for Genetic Control of Mosquitoes may or may not be correct. It is impossible to say without questioning the scientists concerned, which has not been done. Dr Rao could not say much about the formulation of the programme because he came on the scene after this work had been done. (It may be noted in pass-

ing that the remarks on the financial benefits supposed to have accrued to Dr Rao by joining the scheme are contradicted by the Indian Council of Medical Research (ICMR) letter, laying down the terms of Dr Rao's appointment.)

In spite of such errors and misconceptions, we must take due note of the fact that an independent body of men, representing all shades of opinion, has enquired into these projects and has found reasons for apprehension. Although some of these apprehensions may be exaggerated or based on incorrect evidence, there are others, not so prominently emphasised by the committee, which call for attention.

If we accept the proposition that the details of work, the implications of the study, and the safeguards against possible dangers are the responsibility of the expert concerned, it is also incumbent on the authorities to appoint proper and competent experts. Was this done? The committee has rightly pointed out that neither of the two individuals who occupied the office of the Director General of the ICMR (Indian counterpart of the Project Director) was qualified to judge the intricacies of entomological research. This was a serious lapse and though it was soon remedied, by the appointment of an eminent entomologist, Dr T. R. Rao, as an officer on special duty, such an appointment should have been made before any programmes were decided on. This delay may, as the Press Trust of India correspondent claimed, have involved us in programmes of dubious value. Whether this was or was not the case could not be ascertained from the evidence available. But that such a possibility existed is indicative of our failure to maintain adequate vigilance; this is the real danger which needs to be highlighted.

Unfortunately, the defect is a part of a larger malaise, and calls for examination of the defects in our methods of recruitment and appointment which leave a substantial proportion of our most brilliant men and women unemployed, so that they must seek opportunities for work in other countries. And what of our habit of vesting all authority in a top official irrespective of qualifications? But consideration of these points would not be strictly relevant to the issues raised in this report, however important they may be in creating such potentially dangerous situations

It is unfortunate that although the committee has made sincere efforts to examine the issues involved, its conclusions are invalid because of a lack of appreciation of the distinction between the role of the administrator and that of the scientist in research projects.   □

news and views

Hoyle on troubled waters

from John Faulkner

SIR Fred Hoyle (Cockley Moor, Cal-tech, Cornell . . .), the angry young man of British astronomy, turned a surprising 60 recently, and some seventy-five astronomers and astrophysicists met to celebrate the event on July 15–17 at a conference in his honour, "The Frontiers of Astronomy in 1975".

Isola San Giorgio, Venice was the apparently curious but in fact inspired choice of site for a meeting in which past achievements and the at times pessimistic future were surveyed. An attempt to ensure coverage of most areas in which Hoyle had contributed significantly meant that an unusually wide variety of topics were discussed.

In the popular mind, Hoyle is mainly known for his part in cosmological and other arguments. To the professional astrophysicist, however, his most important and enduring achievement must surely be his seminal contributions, going back three decades, to the theory of nucleosynthesis—the production of the elements—in both stellar and cosmological environments. It is worth stressing this point because Hoyle's ideas and their consequences have become such an accepted part of the "obvious" background for present studies that his role has to some extent suffered that curious extinction and obscuration which occurs when "everybody knows that". I have found that not a few professional astronomers are unaware of the 1946 (yes!) paper on the equilibrium process in supernovae and the abundances of the iron peak nuclei. Perhaps they can be forgiven—even Margaret Burbidge (University of California, San Diego) confessed that its importance only hit her six years later. Again, in the early fifties, Hoyle pondered the difficulty of producing the observed abundances of carbon and oxygen in reasonable stellar environments and was led, following Salpeter's identification of the normally unstable ^{8}Be nucleus as an intermediary, to propose the existence of a hitherto unsuspected energy level and its properties in the ^{12}C nucleus. Brilliantly confirmed by Whaling in Caltech's Kellogg Laboratory (despite the earlier scepticism of

his colleagues), this prediction had two important consequences: astronomy had been shown (again) to be a laboratory at large which could lead and stimulate, rather than merely follow, terrestrial physics; and it convinced Fowler (Caltech) to become a nuclear astrophysicist and ultimately led to the massive 1957 work of B^{2}FH. Some young astrophysicists are unaware of the way in which their subject changed from an arid preserve of applied mathematicians into a dynamic part of modern physics. They have Fred Hoyle to thank in large measure for that change.

Fowler discussed recent experimental and theoretical work at the Kellogg Laboratory on the properties of light to medium nuclei. He emphasised that there were many low-lying excited states likely to be important in supernova events, but essentially inaccessible in the laboratory. Work such as Woosley's on the systematics of the nuclei had been very important in this regard, and calculated and experimental charged particle reaction rates were good to factors of ~2 up to ~3 billion degrees. With uncharacteristic pessimism however, he concluded that this global approach to understanding was probably at an end. His colleague C. A. Barnes added to the gloom by remarking that the solar neutrino problem is still with us. The hoped-for escape route by way of a resonance state in ^{8}Be (preventing the ^{8}B neutrinos from being produced) seems to have been killed by no less than three groups working with different reactions (led by Dwarakanath and Kavenagh at Caltech and Parker at Yale). On a brighter note Barnes mentioned that work by Rolfs (University of Toronto) and his Caltech colleagues, measuring reduced widths of states near particle thresholds by direct capture studies, had opened the way for determining cross sections for a whole host of astrophysically important reactions. Gloom returned when he remarked that the trend in cross sections for important carbon and oxygen reactions ran counter to other heavy-ion reactions and were something of a puzzle.

A shaft of Texan sunlight illuminated

the scene when Clayton (Rice University) with customary evangelical enthusiasm presented evidence and arguments that refractory grains formed in supernova or nova ejecta may have survived heating in the early solar nebula (*Nature*, in the press). Concomitantly (*Astrophys. J.*, **199**, 765; 1975), extinct ^{129}I may have decayed in the grains rather than in meteorites, as currently supposed. This could require a major revision of the early evolution of the solar nebula.

In another interesting paper Woosley (Caltech) discussed the nucleosynthesis that can occur in portions of the Universe characterised by relatively high baryon densities (a possibility recently put forward by Hoyle). In contrast with earlier calculations, Woosley was able to produce large amounts of elements heavier than iron, up to $A \sim 100$; indeed, the amount in the range $60 \lesssim A \lesssim 80$ could be embarrassingly greater than in the Solar System. With an intermediate baryon density (as in the Rees "chaotic" models), abundances in this range are quite consistent with those observed in very metal deficient Population II stars, as discussed, among other abundance data, by Pagel (Royal Greenwich Observatory).

On the (possibly) cosmological front, Hazard (Cambridge University) pointed out that the apparent strong degree of density evolution exhibited by QSOs could lead to strong fluctuations in their volume density. An identification programme of Molonglo sources carried out with Murdoch (Sydney University) indeed shows large variations of QSO surface densities for a given redshift in different regions. While it was too early to fall in with the conference spirit and drew sweeping conclusions from grossly inadequate data, the recent discovery (on the same night!) of the two new $z \geqslant 3$ objects at Lick and Steward Observatories encouraged optimism that significant studies might some day prove possible. Ekers (Groningen) recalled Hoyle's 1959 suggestion of a minimum in the angular size–flux density relation in certain cosmological models. Several hundred weak Westerbork sources (down by ~100

on the weakest 3C sources) show such an effect, at last. But the results are not in quantitative agreement with the expectations of simple, non-evolving cosmologies.

In other areas, Wickramsinghe (Cardiff) recalled early work with Hoyle on the nature of interstellar grains. In addition to grains including graphite, a suggestion of a component consisting of silicates covered by crystalline organic polymers is supported by recent data on the melting temperatures of cometary dust. Steigman (Yale University), discussing the interstellar medium as studied through the Copernicus satellite observations, was critical of their earlier interpretation. With Strittmatter and Williams, he has shown that circumstellar HII regions can account for almost all the absorption features observed; hence the bulk of the observed material may be circumstellar rather than interstellar. Solomon (SUNY, Stony Brook) from studying CO emission at $\lambda \sim 2.66$ mm (resulting from $CO-H_2$ collisions) has concluded that most of the interstellar medium in the interior of the galaxy is molecular H_2.

Stellar structure, in which Hoyle also played a large role, was rather under-represented at the meeting. Demarque (Yale University) gave a largely histori-cal review. Faulkner (University of California, Santa Cruz) discussed Hoyle's stimulus on his own horizontal branch work, and recent developments in connection with the possible effects of gravitational radiation on close binary evolution. Arnett (University of Illinois) ruefully showed how six months work since the Dallas meeting (*Nature*, **253**, 231; 1975) had resulted in the calculations of supernova collapse and neutron star formation being pushed about a millisecond further.

Perhaps the most appreciated tribute to Hoyle was that in Rees' (Cambridge University) stimulating talk in which he announced that the main building at the Cambridge Institute of Astronomy would henceforth be known as the Hoyle building. "Being stimulating", said Rees, "can be more important than being right". When Hoyle concluded the meeting with a discussion of his most recent cosmological ideas (after expressing a hope that NASA and the NSF can survive the assaults of democracy) it was clear that many in the audience retained the right to be sceptical But that did not stop them from rising to applaud him for his achievements (as aptly put by the host, Professor Rostagni at the start of the meeting) during the first sixty years of his time on Earth.

electric vector is parallel to the field line, it can shake an electron it meets up and down the magnetic 'wire' and can, therefore, be scattered by it. If, however, its electric vector is perpendicular to the field, it cannot shake an electron as it passes and will emerge without being scattered. Such an aniso-tropic scattering process clearly produces polarisation of the emergent X rays, even if initially the X rays had no net polarisation. Observation that the X rays from the pulsed X-ray sources are strongly polarised would lend strong support to the accreting mag-netised neutron star models. In addition, observation of how such polarisation varies through the pulse cycle would give valuable information about the emission process itself.

A certain amount (though probably less than about 10%) of polarisation is to be expected in the X-ray flux from the non-pulsing sources as well. Material captured from the companion star is likely to have so much angular momentum that it cannot accrete directly on to the compact object. Instead it forms an accretion disk around it. Matter in the disk slowly spirals inward towards the central compact object, releasing energy in the form of X rays as it does so. If the compact object is a black hole, all the observed X rays come from the disk, but if it is a neutron star, about half of them come from a hot thin boundary layer where the disk grazes the stellar surface. In general, in the central region of the disks where the bulk of the X rays is produced, the disk is optically thick and the dominant opacity is due to scattering of the X rays by electrons. Suppose that we view such a disk from an angle, so that its apparent shape is an ellipse, and consider an X-ray photon emerging from the disk towards us. Before it was scattered out of the disk, the most probable direction in which it was travelling is upwards in the disk, that is, in a direction perpendicular to the plane of the disk. Since the electron which gave rise to this last scattering must have been shaken by the photon's electric field in a direction perpendicular to the photon's pre- and post-scattering paths, we see that the photon's electric vector must have been in the plane of the disk and perpendicular to our line of sight Thus photons leaving the disk are preferentially linearly polarised, and the position angle of the polarisation gives us information about the orientation of the disk in space.

In such close binary systems, a fraction (a few per cent) of the X rays emitted by the compact object strike the companion star. Of these, a large proportion is scattered off the stellar atmosphere, while the rest are absorbed and converted into optical and

X-Ray polarimetry in astronomy

from J. E. Pringle

MOST of the known X-ray stars in our Galaxy are believed to be members of close binary systems, in which material from an ordinary star is being accreted on to a compact, X-ray emitting companion. Our present evidence on the nature of these systems comes from studies of the variability and spectral shape of the X-ray continuum and from observations in other parts of the electromagnetic spectrum, in particular at optical and infrared wavelengths. In recent articles, Alan Lightman and Stuart Shapiro (*Astrophys. J.*, **198**, L73–L75; 1975) and Martin Rees (*Mon. Not. R. astr. Soc.*, **171**, 457–465; 1975) have stressed the importance of making observations of the polarisation of the X-radiation from these sources.

The two X-ray stars which display regular pulses, Hercules X-1 and Centaurus X-3, are thought to be rotating, magnetised neutron stars. The material being accreted on to them is funnelled by their strong (about 10^{12} Gauss) magnetic fields and falls on to the star at the magnetic poles. There the energy of infall is released as X rays, and so the poles act as two X ray emitting hot spots on the neutron star's surface. If the magnetic axis is not aligned with the star's rotation axis, then, as the star rotates, first one pole becomes visible and then the other, making the X-ray emission appear pulsed. Although the details of the X-ray emission process are not well understood, it is fairly certain that X-radiation produced in such a strong magnetic field should be strongly polarised. The cyclotron frequency for the electrons is in the X-ray range and the X-ray photons themselves are probably produced by emission at the cyclotron frequency or low harmonics thereof. Such cyclotron emission is itself highly polarised. The X-ray photons then have to find their way out from the emission region at the stellar surface through the column of accreting material which is falling down the magnetic field lines. In a strong magnetic field, the infalling electrons are essentially tied to the field lines like beads on a wire. Consider an X-ray photon travelling perpendicular to the field lines. If its

ultraviolet radiation. Such scattered photons are again likely to be highly linearly polarised. By arguments similar to those above it is clear that the net electric vector of the scattered photons would tend to be aligned with the rotation axis of the binary system. The amount of such polarised scattered X rays received would vary depending on how much of that part of the star that is illuminated by the X-ray source is visible—that is, it would vary with the orbital period. In addition, the smaller the angle between our line of sight and the rotation axis of the binary system, the greater the amount of time for which the illuminated part of the star is visible and hence the smaller the size of the variation. Thus, in principle, such an observation could tell us the inclination of the binary orbit to our line of sight. Because such a small fraction of the emitted X rays actually strikes the companion star, such an effect would, however, be difficult to detect.

X-ray polarimetry is at present in its infancy, but it seems likely to grow to maturity over the next few years. In the past, the best-known argument for X-ray polarimetry has been that it is often symptomatic of non-thermal synchrotron-type emission. Drs Lightman, Shapiro and Rees have drawn attention to other sources of X-ray polarisation and, more importantly, have shown how X-ray polarimetry can be used as a testing ground for theoretical models, and as a means of probing the structure, of the binary X-ray stars.

Ozone over Britain

from Peter D. Moore

IT is no longer possible for the British to be complacent about ozone pollution Though Los Angeles is still the worst hit area of the world, having occasional peaks of 70 p.p.h.m. of ozone in its air (Marx, *Science*, **187**, 731; 1975) and having at least once reached 99 p.p.h.m., the problem is far more widespread : even in Melbourne peaks of 28 p.p.h.m. have recently been recorded.

In the United States, ozone is regarded as by far the most damaging of air pollutants to plants. In New Jersey, Pell and Brennan (*Environ. Pollut.*, **8**, 23; 1975) consider that $128,000 worth of damage was caused to agricultural and ornamental plants in 1972, and estimated that losses of over $35,000,000 have been made for California. Ozone is not yet considered an economic threat to British agriculture.

In 1972, Atkins *et al.* (*Nature*, **235**,

372; 1972) demonstrated the production of atmospheric ozone over southern England and later Derwent and Stewart (*Nature*, **241**, 342; 1973) published data for central London in which they found daily peaks of 8–12 p.p.h.m. Recently Cox *et al.* (*Nature*, **255**, 118; 1975) have concluded that a proportion of southern England's ozone may have been transported considerable distances, of the order of 100–1,000 km. It seems clear, therefore, that Britain has an ozone pollution problem, though it is not entirely of her own making.

Bell and Cox (*Environ. Pollut.*, **8**, 163; 1975) have now published data, obtained in 1972, concerning plant damage by ozone in Britain. They exposed the ozone-sensitive variety of Bel-W3 of tobacco to the atmosphere in Ascot (32 km west of central London) and at the same time monitored the air for ozone. They observed the characteristic symptoms of ozone toxicity (necrotic spots on the leaf surface) and their atmospheric measurements showed that ozone levels had risen above 4 p.p.h.m. on 322 occasions between July 4 and September 15 and above 6 p p.h.m. on 83 occasions. On one occasion a level of 10 p.p.h m. was maintained for 5 h. (The United States air quality standard of the Environmental Protection Agency is 8 p.p.h.m. for one hour.) Bell and Cox confirmed that there was a strong positive correlation between the degree of leaf damage and the length of time for which each test plant had been exposed to ozone levels in excess of 4 p.p.h.m. More resistant tobacco varieties showed very low levels of damage and no significant correlation with ozone exposure; no correlation at all was demonstrable with sulphur dioxide concentration.

These results show that diurnal peaks in ozone, at levels which could be injurious to both man and plants, are not restricted to central London, but could be occurring over a much wider radius. No records have yet been reported of ozone damage to crops or to wild plants in Britain but, as Bell and Cox point out, growth may be suppressed without symptoms being visible.

Recent experiments on a number of herbaceous plants in America, however, by Harward and Treshow (*Environ. Cons.*, **2**, 17; 1975) suggest that visible symptoms and growth reduction are rare at levels of 5–7 p p h.m. ozone for about 2 h per day, but become apparent in some species when levels of 15 p.p.h.m. are used. In August 1973, levels as high as 17 p.p.h.m. were recorded at Harwell, 80 km west of London, so perhaps visible damage will soon make itself apparent and we can recognise that we do indeed have an ozone problem.

Agglutination of tumour cells

from Robert Shields

MUCH research effort has gone into investigations of why plant lectins often agglutinate tumour cells to a greater extent than their normal counterparts. As with all generalisations about tumour cells, enhanced agglutination is not an invariant property of transformation. It is, however, sufficiently widespread in the model systems used by cell biologists to have promoted intensive study

Using the lectin concanavalin A (Con A) it has been shown that agglutinability of some lines of cells is directly related to their tumorigenicity when injected into animals (Inbar *et al.*, *Nature new Biol.*, **236**, 3; 1972). Also, cells transformed with temperature-sensitive tumour viruses exhibit high agglutinability at the permissive temperature (where the transformed phenotype is expressed) and lowered agglutinability at the non-permissive temperature (Burger and Martin, *Nature new Biol.*, **237**, 9; 1972; Eckhart *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **68**, 283; 1971). The experiments show that high agglutinability is closely associated with oncogenic transformation This idea was considerably strengthened by experiments that used a variety of selective pressures to produce revertants of tumour cells with approximately normal behaviour. The revertants are less agglutinable and less tumorigenic than their transformed parents (Pollack, *In Vitro*, **6**, 58; 1970; Pollack *et al.*, *Proc. natn. Acad. Sci. U.S A.*, **60**, 126; 1968). One of the best pieces of evidence for the close linkage of agglutinability and transformation is from work carried out by two separate groups (Culp and Black, *J. Virol.*, **9**, 611; 1972; Ozanne and Sambrook, *Lepetit Colloq.*, **2**, 248; 1971) on revertants. They used the fact that agglutination of tumour cells with high doses of Con A leads to cell death. After treating transformed cells with high concentrations of the lectin and isolating surviving cells, they were able to establish cell lines with reduced agglutinability and a more normal phenotype.

It has recently been suggested that tumour cells are highly agglutinable because they have more microvilli on their surfaces and that the presence of these microvilli is regulated by cyclic AMP (Willingham and Pastan, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 1263; 1975). These workers suggest that microvilli help agglutination by bringing larger areas of cells' surfaces into close apposition, allowing them to be cross-linked by lectins. They suggest that it is the low level of cyclic AMP

in tumour cells which allows formation of microvilli and hence agglutination.

Using dark field microscopy these workers found that the transformed cells examined had many hairlike microvilli on their surfaces, and normal cells (in this case 3T3 cells) had none. But when 3T3 cells rounded up in mitosis or were treated briefly with trypsin numerous microvilli could be seen. These are precisely the conditions where cyclic AMP levels are low and the cells agglutinate readily. The concept that cyclic AMP could regulate agglutinability through microvillus formation was tested directly by incubating transformed cells for extended periods with dibutyl cyclic AMP, then examining their surface for microvilli and measuring agglutinability. After 24 h treatment the cells had few microvilli and were far less agglutinable. But the decreased agglutination of tumour cells after prolonged incubation with dibutyl cyclic AMP may have more to do with expansion of the G2 phase of the cell cycle (Smets, *Nature new Biol.*, **239**, 123; 1972) where transformed cells are less agglutinable (Smets and deLey, *J. Cell Physiol.*, **84**, 343; 1974) than any genuine reversion to a more normal phenotype. Also dibutyl cyclic AMP has been reported to affect the syntheis of some cell surface components (Goggins *et al.*, *J. biol. Chem.*, **247**, 5759; 1972), and it is possible that it interferes with agglutination through its effect on these molecules rather than by any direct action on microvilli.

Better evidence for the direct role of cyclic AMP in agglutination comes from the use of prostaglandin E_1 or the phosphodiesterase inhibitor isobutyl methyl xanthine. Brief treatment of transformed cells (L929) with either compound leads to a rapid increase in cyclic AMP as well as a decrease in microvilli and a decrease in agglutination. When the cells are washed free of these componds cyclic AMP levels drop and microvilli and agglutinability return.

The idea that transformed cells are agglutinable because they have numerous microvilli is only the most recent of a long series of explanations, most of which have been oversimplified or erroneous. However, the theory does have the merit of being easy to test. It should be pointed out that not all tumour cells are more agglutinable than normal cells and not all cells that agglutinate have numerous microvilli. There is no reason to suppose that a single difference between cells is the reason for the difference in their agglutinability. It seems far more likely that agglutination is a very complex process involving factors such as microvilli, surface charge and density and mobility of lectin receptors and

only in a few model systems will one factor predominate. Perhaps only when agglutination is understood in simple cells such as the erythrocyte will factors that control agglutination in more complex cells be understood.

Around the world ballooning for dust

from David W. Hughes

BALLOONS have been used by scientists ever since the pioneering work of Etienne and Joseph Montgolfier in 1783, either to investigate the atmosphere or to lift apparatus above most of it. Modern plastic balloons provide an inexpensive vehicle that can lift thousands of kilograms above 99% of the atmosphere (to heights of ~33 km) or hundreds of kilograms above 99.9% (to ~50 km) and can carry the weight there for days.

One of the latest experiments, named Magellan, is jointly organised by Wlochowicz of the National Research Council of Canada, Ottawa and Hemenway, Hallgren and Tacketh of the Dudley Observatory, Albany, New York. Preliminary results were given at the IAU Colloquium no. 31 on Interplanetary Dust and Zodiacal Light recently held at Heidelberg. Magellan is designed to fly at an altitude of about 30 km and to collect relatively large solid particles, diameter 50–300 μm (mass 10^{-7}–5×10^{-5} g), as they fall through the atmosphere.

As the flux of particles is low the experiment must have a large collecting area exposed for a long time, but the particles must be concentrated in a much smaller area for easy counting, photography and chemical analysis. To do this the Magellan balloon payload has a vertical conical mylar funnel (like an umbrella) the wide end being uppermost and 50 m² in area. The funnel apex has an angle of 60° and particles falling into the funnel slide down the sides into a sampling pan at the bottom. The payload has three sampling pans which can be exposed for pre-set periods of time and then sealed. The funnel is hung from a 300 m nylon line below the balloon to minimise contamination. The payload weighs 385 kg and contains an electronics package for tracking and for activating the cut-down mechanism.

In two engineering flights from Australia using a 20 m diameter super-pressure balloon one payload went all the way round the world and was cut down and eventually recovered within 15 km of the launch site. The second payload was not so well behaved, circling the Southern Hemisphere twice and staying up for 210 days before

finally falling into the Pacific ocean. Two short-duration flights from the NCAR Balloon Flight Station at Palestine, Texas, showed that about nine particles per hour were collected by the system. These particles are now being analysed to give incident flux, mass distribution and composition.

As in all new techniques there are problems. First, the particles can have different origins. They could be micrometeoroids (particles which do not reach boiling point on atmospheric entry and are retarded and fall slowly to Earth); they could be fragments of much larger meteoroids which disintegrated on entering the atmosphere; or they could be terrestrial contaminants which have fallen off the balloon or been blown up to 30 km from lower down Only the micrometeoroid flux is known as this can be inferred from spacecraft data. The second problem is political. It is hoped that the work will not be hindered by nations reticent about balloons flying across their territory and that multinational cooperation will soon allow Northern Hemisphere flights to start.

Two-scale mantle convection

from Peter J. Smith

ALTHOUGH convection currents in the Earth's mantle have been the subject of extensive theoretical study and speculation, experimental investigations of convective models have been surprisingly few. Yet as Richter and Parsons (*J geophys Res.*, **80**, 2529; 1975) point out, laboratory studies have a crucial part to play in testing the stability of analytical and numerical models, especially of three-dimensional systems. In spite of previous work suggesting that the conditions for stable convection may be quite restrictive, the question of stability is all too frequently ignored—a situation which Richter and Parsons claim, somewhat wryly, "may in part be responsible for the proliferation of mantle convection models that bear little resemblance to one another".

Some years ago, for example, Busse (*J. Math Phys.*, **46**, 140; 1967) showed that two-dimensional convective rolls in a fluid layer between rigid stationary boundaries are only stable for Rayleigh numbers less than 13 times critical. At higher Rayleigh numbers, a second set of rolls develops at right angles to the original one, giving a rectangular pattern when viewed in plan (bimodal convection); and at Rayleigh numbers about 100 times critical, instability develops, giving a plan pattern which

resembles spokes radiating out of central upwellings or downwellings (multimodal convection). But in spite of this and other evidence, many workers have assumed stability for convective models of the upper mantle where the Rayleigh number is probably several hundred times critical. According to Richter and Parsons, for a model governed by nonlinear dynamics it is just not sufficient to find a solution satisfying the Rayleigh convection equations and the boundary conditions. The solution must also be shown to be physically possible; and only laboratory experiments can easily provide this critical information.

A good illustration of this point is provided by Richter's (*J. geophys. Res.,* **78**, 8735; 1973) own attempt to account for the pattern of oceanic heat flow. The high heat flow anomalies over active oceanic ridges can readily be explained in terms of large-scale convection. This is the well-known system in which mantle material rises at ridges, the seafloor spreads laterally and descends at oceanic trenches, and the cycle is completed by return fluid flow in the upper mantle. Much more difficult to understand, however, is the approximately constant and uniform heat flow through ocean floor older than a few tens of millions of years. Very little of the heat involved here can be produced in the oceanic crust itself which contains too few radioactive sources. Most of it (about 1 μcal $cm^{-2}s^{-1}$) must therefore come up from the mantle. But this raises the problem of how the mantle can transport so much heat. Calculations based on the available knowledge of upper mantle composition and physical properties show that neither conduction nor radiation is sufficient.

To overcome this difficulty, Richter proposed the existence of small-scale convection beneath the plates—convective cells having a horizontal scale comparable with the depth of the fluid flow region but smaller than the plate dimensions which characterise the horizontal segments of large-scale convection. In other words, he envisaged two coexisting horizontal scales of motion in the upper mantle (the vertical scales are the same)—one required by the moving plates and one by heat flow through old ocean floor. The principal result to emerge from analytical and numerical studies of such a system was that the large-scale convection suppresses small-scale convective rolls having axes perpendicular to the large-scale flow (transverse rolls) and, in time, replaces them with rolls having axes in the direction of large-scale flow (longitudinal rolls). Unfortunately, however, this analysis was strictly valid only for Rayleigh numbers slightly higher than critical. So does it, or does

Same species, different profiles
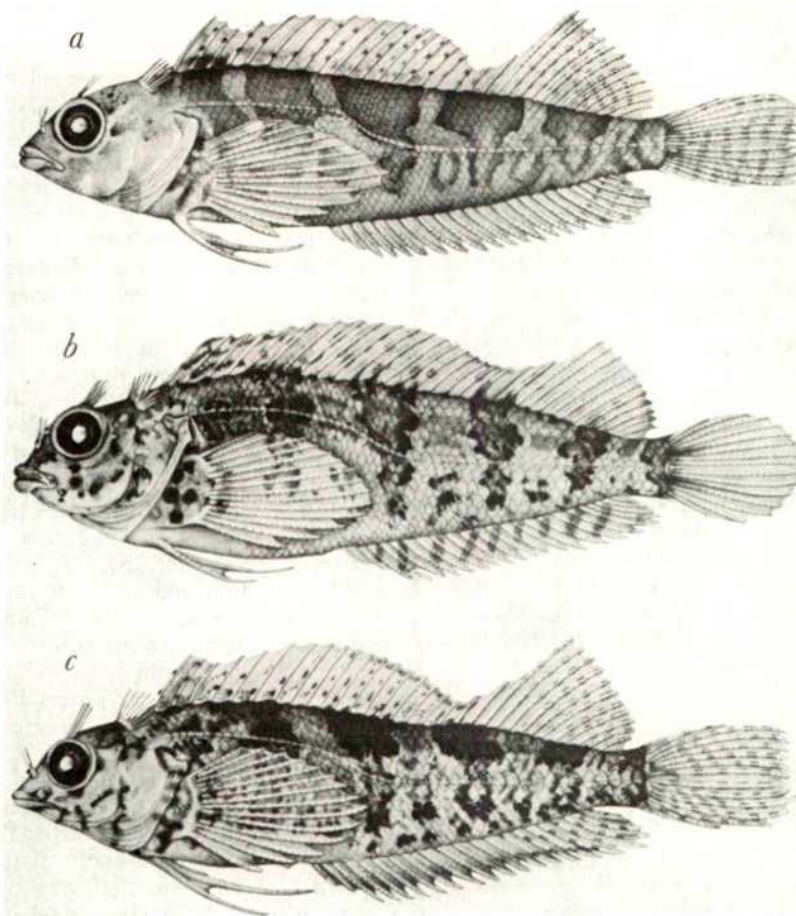
Malacoctenus triangulatus females. *a*, from Hog Island, Bahamas; *b*, from Trinidad; *c*, from Brazil. Drawn by J. R. Schroeder; from *Smithsonian Contributions to Zoology* no. 200 by Victor G. Springer and Martin F. Gomon; Smithsonian Institution, 1975.

it not, also apply to Rayleigh numbers more typical of the mantle?

This is where laboratory work is crucial; and the results of a series of experiments designed to study the interaction of small-scale and large-scale convection have now been reported by Richter and Parsons. Briefly, these are that irrespective of the initial stable state of small-scale convection (transverse rolls, bimodal convection or multimodal convection, depending on Rayleigh number over the range 3.0×10^3–6.8×10^5), the imposition of large-scale convection ultimately changes the form of the small-scale flow to longitudinal rolls. The experimental data thus both demonstrate the plausibility of two-scale convection and confirm that the earlier theoretical results also apply at high Rayleigh numbers.

They also enable a time scale to be applied to the development of the longitudinal rolls under different conditions. Assuming that the Rayleigh

number of the sub-oceanic upper mantle approaches 10^6 and that convection extends down to the 650-km seismic discontinuity (assumptions which Richter and Parsons attempt to justify in detail), the experiments suggest that where the plate velocity is 10 cm yr^{-1} the small-scale convection should become aligned as longitudinal rolls within 20–50 Myr. On the other hand, for a plate velocity of 2 cm yr^{-1} the time scale increases to several hundred million years. Taking into account typical lengths of time between major changes in plate motion, these results suggest that longitudinal rolls within the small-scale convection system should only develop where rates of plate motion are high.

Richter and Parsons have thus combined theory, experiment and some observation (for example, of heat flow) to produce an internally consistent two-scale convection model. But can these ideas be tested, or are they destined to remain unprovable hypotheses? The

problem here is the shortage of observational constraints that can be related directly to convection rather than just to the rigidity of the plates. Two possibilities suggested by Richter and Parsons, however, are free-air gravity anomalies and residual elevation anomalies (differences between measured ocean floor depths and the corresponding depths to be expected as the result of the cooling of the lithosphere as it moves away from the ridge). As far as the roll-like pattern of convection is concerned, the best place to look for such evidence would probably be the Pacific where the plate velocity is relatively high and where no significant change in spreading direction has apparently occurred for 40-45 million years. But the region in which anomalies have been most closely observed so far is the North Atlantic where the slower spreading rate makes roll-like features less likely to be observed (nor are they) and thus where the small-scale convection pattern is more likely to take the form of upwelling and downwelling spouts. This mismatch is unfortunate, but with luck only temporary.

Peanuts and cancer

from Arie J. Zuckerman

THE geographical pathology of primary cancer of the liver presents intriguing features. Liver cell cancer is rare in North America, Europe, the Soviet Union and Australia and it seems to be relatively infrequent in Central and South America. In contrast, hepatocellular carcinoma is common in many communities in Africa and South-East Asia and probably less so in Japan. In some parts of the tropics, liver cell cancer is the commonest type of cancer in adult males and it is often, though not always, found in patients with cirrhosis. Epidemiological and experimental evidence is accumulating to implicate natural hepatocarcinogens as contaminants in foodstuffs and hepatitis B virus in the aetiology of primary liver cancer.

The carcinogenic potential of a number of mycotoxins and pyrrolizidine alkaloids is now well established in experimental animals. The aflatoxins form a group of closely related substances produced by certain strains of moulds of *Aspergillus flavus-oryzae*. These substances have been shown to have marked toxic and carcinogenic effects on the liver and other tissues of experimental animals. The cytotoxicity of aflatoxins on human embryo liver cells in cultures has also been demonstrated; aflatoxin inhibited DNA, RNA and protein synthesis in such cultures (Zuckerman *et al., Br. J. exp.*

Path., **49**, 33; 1968). The order of toxicity of aflatoxin B_1 and G_1 in human liver cells was similar to the order of carcinogenicity in rats.

The mould *Aspergillus flavus* readily grows on groundnuts (peanuts) and grains when they are stored traditionally in the open, in hot, humid conditions. Indeed approximate relationships have been reported between aflatoxin contamination of market food samples and the incidence of primary liver cancer in Uganda, Swaziland and Thailand. Peers and Linsell (*Br. J. Cancer,* **27**, 473; 1973) found a significant correlation in the incidence of liver cell cancer and the amount of aflatoxin actually consumed in a study carried out in three different altitude areas in the Muranga district of Kenya. In the low altitude area, below 5,250 foot above sea level, the incidence of liver cancer was greater and the aflatoxin levels in the food samples were higher. Van Rensburg and colleagues (*S. Afric. J. Med.,* **48**, 250a; 1974) have also shown that aflatoxin consumption by Africans living in the Inhambane district of Mozambique was remarkably high, correlating well with the high prevalence of liver cancer in that area. Peanuts and, in some areas of Africa, maize form the staple diet of Africans and the consumption of food contaminated with fungal matabolic products is likely to be high. Yet it would be prudent to note that the quantity of aflatoxin that would need to be ingested by an individual in the form of contaminated peanuts, assuming that the dose is similar to that needed to cause experimental necrosis of the liver seen in animals, is in gross excess of that likely to be consumed, amounting to several kilograms per day (Higginson, *Tumours of the Liver,* Pack and Islami; Berlin, 1970).

The apparently high incidence of infection with hepatitis B virus in chronic liver disease and especially macronodular cirrhosis and primary liver cancer in many regions in Africa and South-East Asia also implies the possibility of some form of an aetiological association. That a strong correlation has been found in some studies cannot be denied, but there are also a number of conflicting reports from different regions. The most recent study by Bagshawe and her colleagues in Kenya (*Br. J. Cancer,* **31**, 581; 1975) revealed that in the Muranga district (see above) no significant differences were found in the prevalence of hepatitis B surface antigen (3.6%) between the low altitude areas, with a relatively high incidence of primary liver cancer, and the high altitude area (2.7%) with a low incidence of the tumour.

Liver cell cancer is probably the cumulative result of many factors, and the roles of viral and parasitic infec-

tion, chemical carcinogens, nutritional and other environmental factors remain to be determined.

QSOs still problematical

from R. F. Carswell

THE origin of absorption lines in some QSOs remains something of a puzzle. Perhaps the only thing that is clear is that there are absorbing clouds some distance from the QSO, which have redshifts which are usually less than that of the emitting object itself. The velocity differences are usually less than about one-tenth the speed of light, but in one well established case the velocity is about half the speed of light. Even so, the redshifts of the QSOs in which these absorptions are seen are so high that, even with these large velocities relative to the QSO, the clouds are still receding from us. Study of the absorption line regions is clearly important, since if they are intervening galaxies then they can give us some idea of the nature of galaxies during the early Universe, and if they are intrinsic to the QSOs then they must be telling us something about the properties of the QSOs themselves. There are a number of problems in interpreting the observational data however, which leave the situation unclear.

The first major problem is that of identifying the redshifts of the absorption clouds, given a spectrum which may contain anything up to 200-300 absorption lines. A single QSO may have many absorbing clouds, at many very different redshifts, each with differing excitation properties so that it is not always possible to say definitely what lines, or redshift systems, must be present. What is usually done in the case of a newly discovered absorption line QSO is to try all reasonable candidate lines at all possible redshifts, and see if any physically consistent, and statistically significant, absorption systems are present. Often this is done on a fairly subjective basis, but a computer program developed by Aaronson, McKee and Weisheit (*Astrophys. J.,* **198**, 13; 1975) may help bring a consistent approach to the subject. The authors have analysed all previously published data on absorption line QSOs looking for candidate absorption systems using a coherent set of atomic physics and ionisation equilibrium criteria, and checked the probability of finding such a system by chance. They show that the number of well-established systems is in fact much smaller than had previously been believed, notably in very absorp-

tion rich spectra such as PHL957 and 4C05.34 where there is a high probability of chance identifications This still leaves a large number of unidentified lines, which may be due to optically thin clouds with too few absorption lines to be sure of their reality in individual cases, but which together account for most of the features in the spectrum.

Aaronson *et al.* also examine the question as to whether or not the probable absorption redshifts they have are likely to have arisen from intervening galaxies or matter ejected from the QSO. Perhaps not surprisingly, with only eight absorption line QSOs for which good spectral material is available, it seems that the available data are consistent with either picture, though they suggest that the intrinsic hypothesis might be slightly favoured. To verify this we must await additional material on new absorption line QSOs

The question as to whether or not there are intervening galaxies between us and some QSOs depends to some extent on how distant the QSOs are, and this brings us to consideration of the nature of the redshift. It is generally believed that the high redshifts observed in QSOs are a result of nothing more than the general expansion of the Universe that seems well established from studies of normal galaxies at much lower redshifts. High redshifts are seen in QSOs only because they are intrinsically bright objects and can therefore be seen to much greater distances. But in spite of the wide acceptance of this view, some astronomers contend that there is really not sufficient evidence to say that there may not be some other explanation, for all or part of the high QSO redshifts, perhaps involving some physical principles of which we have no knowledge at present.

Given that we have no model of QSO behaviour under such circumstances, such a hypothesis is very difficult to test, but some headway can be made if we believe that some QSOs are physically associated with, for example, galaxies of much lower redshift. If this is true, then there could be an excess of QSOs near bright galaxies compared with the numbers we would expect on the basis of estimates of the number density of QSOs in the sky that have been made by Sandage and Luyten (*Astrophys. J.,* **155**, 913; 1968) and others A few years ago Burbidge, Burbidge, Solomon and Strittmatter (*Astrophys J.,* **170**, 233; 1971) found that in fact there were more QSOs identified from the 3C (third Cambridge) radio catalogue lying within 7' of bright galaxies than could be due to chance, and subsequently another 3C QSO was found close to such a galaxy. This was fairly

strong evidence, on the face of it, that QSOs had some intrinsic redshift. Subsequent studies of identifications from other radio catalogues did not show the same behaviour, though, and so after a while the result looked more as if it could have been due to chance apparent associations.

Recently, however, Arp, Baldwin and Wampler (*Astrophys. J.,* **198**, L3; 1975) have found two new radio-quiet QSOs near bright galaxies, and so now there are a total of four such known. On the basis of the estimate of the number of QSOs brighter than 19 mag the areas around several thousand galaxies would have to be searched to find four QSOs if they are randomly distributed, while the number of such searches made has been estimated by the authors to be about twelve. This suggests, then, either the estimates of numbers of radio-quiet QSOs are far too low or the associations are likely to be real. There are well-known dangers in drawing conclusions from statistics performed after the event, however carefully the work is done, so it is to be hoped that further searches in neighbourhoods of galaxies will be performed to see if the results are confirmed for a new sample.

Starting at the end
from R. Kamen

An EMBO-Inserm workshop on the *in vitro* transcription and translation of viral genomes was held on July 15–18 at Grignon, near Paris.

THE RNAs of picornaviruses such as polio and EMC have long been the paradigms for eukaryotic polycistronic messenger RNAs. These large RNAs encode a single nascent polyprotein which is cleaved to generate stable viral polypeptides, prompting the general hypothesis that eukaryotic mRNAs do not contain functional internal signals for the initiation of protein synthesis. The recent development of various plant and animal systems for *in vitro* protein synthesis and their successful application to the translation of a wide variety of viral messengers has provided a wealth of data generally supporting this hypothesis The picornaviruses, however, now appear as one special case of a more general mechanism involving mRNA as well as protein processing The results obtained in several laboratories, discussed at the Grignon workshop, fit a common pattern and experiments presented on the role of 5' terminal 'capping' (see *Nature,* **255**, 9; 1975) in translation may explain why eukaryotic ribosomes start at the ends of mRNA molecules.

The process common to many of the viral systems discussed is the following: the 5' terminal portion of a viral polycistronic mRNA functions efficiently in encoding a viral protein or polyprotein, whereas the 3' terminal region remains largely silent even though it contains the sequences which encode a further viral polypeptide. A smaller viral mRNA, comprising the 3' terminal cistron, must be generated (either by RNA cleavage or by selective transcription) to activate translation of the second protein. P. Kaesberg's (University of Wisconsin) results on brome mosaic virus (BMV) well illustrate this mechanism. BMV is a multicomponent plant virus comprising three distinct virions containing four different RNAs. RNAs 1 and 2 each have their own particles, while RNA 3 $(0.7 \times 10^6$ daltons) and RNA 4 $(0 3 \times 10^6$ daltons) share the third type of particle. When translated in the wheat germ system, RNAs 1 and 2 prove to be monocistronic messengers coding for large proteins of size consistent with their RNA molecular weights; thus the proximity of the initiation point to a 5' end is assured by the genome segmentation. RNA 3. however, includes at its 3' end all of the sequences of RNA 4 When translated *in vitro*, RNA 3 encodes one major polypeptide (called 3a) unrelated to the BMV coat protein, while RNA 4 is an efficient messenger for the coat protein. Thus RNA 3 contains one active 5' proximal start signal and a second largely inactive internal start signal which is unmasked in the generation of RNA 4. Competition studies showed that the second start in its

active state, has in fact higher affinity for ribosomes than the start at the 5′ end of RNA 3. Two alternative explanations for the dramatic alteration in efficiency of this initiation site between RNAs 3 and 4 were discussed: either the structure of RNA 3 blocks internal initiation, or a modification at the 5′ end of RNA 4 is involved in ribosome binding. Isolation and sequencing of a fragment of RNA 4 which specifically binds to ribosomes led Kaesberg to opt tentatively for the second alternative· the sequence is m⁷GpppGUAUUAA-UAAUG . . . followed by the codons for the first ten amino acids of the coat protein. A. J Shatkin (Roche, New Jersey) pointed out in discussion that the UAAU tetranucleotide of this sequence is complementary to the common 3′ end of eukaryotic 18S rRNA recently determined by Steitz, Shein and Delgano. Thus the mechanism of ribosome recognition may be in part similar to that used in prokaryotes. Results presented by M. N. Thang (Institut Biologie Physico-Chimique, Paris) and by L. van Vloten-Doting (State Universitv. Leiden). obtained with the small RNAs of the related alfalfa mosaic virus, although more preliminary, concurred with Kaesberg's conclusions on BMV.

T Hunt (University of Cambridge) discussed the *in vitro* translation of tobacco mosaic virus (TMV) RNA. He and his colleagues find that the 2.2 × 10⁶ dalton genomic RNA encodes overlapping 160,000 and 140.000 dalton proteins, but no coat protein. A small viral RNA (0.25×10⁶) found in infected leaves, comprising the 3′ end of the total RNA, is a very efficient messenger for TMV coat protein in several *in vitro* systems. Among the plus-stranded RNA animal viruses. two groups—I. Kennedy (University of Warwick), and L. Kääriäinen (University of Helsinki)—reported experiments with Semliki Forest virus consistent with the notion that the 42S genomic RNA encodes large nonstructural proteins while the 26S viral mRNA found in infected cells (comprising the 3′ terminal third of the 42S) is the mRNA encoding a different single polyprotein which is efficiently processed *in vitro* into the four viral structural proteins.

The mechanism also functions in DNA animal viruses. A. Smith (ICRF, London) and C. Prives (Weizmann Institute, Rehovot) agreed that the 19S late messenger either of SV40 or polyoma virus encodes the minor virion structural protein(s), but is an inefficient messenger for the major capsid protein (VP1). The 16S late messenger, which at least in the case of polyoma virus comprises the 3′ terminal half of the 19S mRNA, is the messenger for VP1 synthesis *in vitro*. Y.Aloni

recently reported at the European Tumour Virus Group meeting that the 19S SV40 mRNA is cleaved to 16S in the cytoplasm, and several groups have found that it has an m7G capped 5′ end.

Evidence implicating 5′ terminal methylation in ribosome recognition was presented for VSV RNA by H. P Ghosh (McMaster University, Ontario) and in detail for several messengers by Shatkin. The latter reiterated his published evidence that non-methylated reovirus or VSV mRNA functions poorly in wheat germ or mouse L cell *in vitro* systems, but can be stimulated by *in vitro* methylation in the presence of S-adenosylmethionine in either system. He also showed that chemical

removal of the terminal m7G from these viral messengers or from rabbit globin mRNA destroys messenger activity. Most interesting were direct experiments on messenger binding: the 7mG of reo messenger is required for binding to wheat germ ribosomes A 35-nucleotide long fragment of reo messenger containing the 7mG 5′ end, which rebinds to ribosomes, was isolated from sparsomycin initiation complexes after nuclease digestion. 7mG has also been found in the globin mRNA ribosome binding site. Finally, Shatkin described experiments in which the 7mGpppGᵐ 5′ terminal dinucleotide of Reo virus was extended with various mixed or homopolymers and assayed for ribosome binding. Only the

New blood parasites for old

from F. E. G. Cox

THE malaria parasites belong to the protozoan sub-order Haemosporina which also contains a number of other blood parasites. From the earliest days of parasitology these have received considerable attention and it comes as something of a surprise to discover that until recently a whole new family has existed unrecognised in South American lizards (Lainson *et al., Int. J. Parasit.*, **1,** 241; 1971; *Parasitology*, **68,** 117; 1974; *Parasitology*, **70,** 119; 1975). This family is the Garniidae and its importance has grown since its discovery in 1971 partly because it looks as if it is going to contain a large number of species and partly because of the implications of the various life cycles which its members undergo. The interpretation of these life cycles throw doubts on current ideas regarding the nature of reptilian malarias and on the evolution of the malaria parasites as a whole. So far, two genera have been described, *Garnia* with six species and *Fallisia* with four.

All the other members of the Haemosporina undergo asexual division, or schizogony, in fixed cells and the malaria parasites, in addition, multiply in circulating blood cells. In the Garniidae, however, schizogony occurs only in blood cells: in red cells in *Garnia* and white cells in *Fallisia*. Some species of *Fallisia* occur in neutrophils and thrombocytes, cells which have not been utilised by any other parasites. Since the discovery of the exoery-throcytic stages of the malaria parasites it has been widely accepted that schizogony in the blood evolved secondarily but this belief will now

have to be reassessed. In addition, all the descriptions of malaria parasites of lizards will have to be re-examined as Lainson and his colleagues have found that multiple infections with members of the Garniidae and with true pigmented malaria parasites are common and this may well have confused the original descriptions of different species. Telford (*Int. J. Parasit.*, **3,** 829; 1973) has claimed that some members of the Plasmodiidae, the malaria parasites, are not pigmented but the recent work indicates that there is no need to redefine this family in order to include non-pigmented forms.

While new blood parasites are being discovered in lizards it is likely that some widely accepted species (and even genera) in birds will have to disappear. Box (*J. Protozool*, **22,** 165; 1975) has found that infections with the intestinal eimerian *Isospora serini* in canaries results in the appearance of extra-intestinal stages in blood monocytes, something that does not happen with another species *I. canaria*. Box notes the similarities between these extra-intestinal stages of *Isospora* and *Lankesterella* spp. and other eimerian parasites which occur in blood cells. This is an extension of the discovery a few years ago that *Toxoplasma gondii* in many mammals is an aberrant *Isospora* sp. of cats.

The status of all non-pigmented blood parasites will have to be looked at critically in the light of the separate studies of Box and Lainson and his colleagues and the parasites of mammals cannot be allowed to escape the scrutiny.

m7GpppG™poly(A,U)   derivative, which should contain molecules with the . . . UAAU . . . sequence complementary to the 3′ end of 18S ribosomal RNA, showed significant interaction with 80S ribosomes.

Several observations must be mentioned which are in apparent conflict with the appealing idea that eukaryotic ribosomes only start at ends because they interact with 7mG Hunt mentioned evidence from TMV and M. Pranger (University of Utrecht) and Kääriäinen gave evidence from Semliki Forest virus, that there is more than one initiation site in the 5′ terminal portion of their respective mRNAs. R. Gesteland (Cold Spring Harbor), in discussing elegant experiments showing that yeast suppressor tRNAs work in mammalian cell-free systems, reconfirmed the observation that an internal cistron of bacteriophage Qβ RNA is translated accurately by eukaryotic ribosomes and initiation factors Moreover, to return finally to the picornaviruses, these RNAs can be translated in vitro although, as discussd by P Fellner (Searle, High Wycombe). 7mG cannot be detected at their 5′ ends.

Insectiverous grouse

from our Animal Ecology Correspondent

THE traditional management of heather moorland for red grouse production centres on regular burning of the vegetation so as to encourage sprouting of new green shoots. Such new growth is rich in nitrogen and phosphorus, elements now known to be correlated with performance of grouse populations (Moss, J. Anim. Ecol , 38, 103; 1969). The practice of burning seems right and proper since heather forms the bulk of the diet of adult grouse. But most species of animals are opportunist feeders—the grouse is no exception—and adherence to too rigid a management creed may deny some moors the extra grouse they could produce.

Different grouse moors do not have identical vegetation. About two-thirds of the total acreage in Britain is low lying and fairly dry. The remaining one-third lies at high altitude and the high annual rainfall it sustains keeps its deep peaty surface waterlogged almost the whole year through. Recent studies by Butterfield and Coulson on one such blanket bog moor reveal that adult grouse supplement their diet with quite considerable amounts of insect food (J. Anim. Ecol., 44, 601; 1975). The habitat is just right for craneflies, chiefly Tipula subnodicornis and Molophilus ater, and at the peak of emergence their densities may reach 15 m⁻²

and 400 m⁻² respectively. Adult male craneflies do not fly strongly and the females have no wings at all. They represent to the grouse an additional source of food almost as easy to harvest as the heather tips upon which they spend most of their adult lives. That adult grouse can develop a specific search image for craneflies is clear from the observation that one grouse shot in October 1970 had the remains of 495 adults of the rare T. gimmerthali in its stomach. This is more than the total number of individuals caught by British entomologists since the end of the nineteenth century.

Observations during May, June and July of fresh droppings taken from established grouse territories on damp moor revealed that the percentage containing insect material varied from 98% at the time of maximum emergence to 8% at the end of the hatching season. Most droppings contained plant material but in a small number the amount fell to less than a half of the total contents. Both species of cranefly were eaten by adult grouse, but chicks took considerably more of the small M. ater than they did of the larger T. subnodicornis For adult grouse the reverse was true. By contrast, droppings from a dry moor were almost totally devoid of insect remains.

What is of great interest is that weight for weight, T. subnodicornis contains nine times more nitrogen, seven times more phosphorus, six times more sodium and twice as much potassium as heather shoots. M. ater is richer, too, in these elements but less so than its larger relative. The importance of these elements in grouse nutrition is shown by the choice by breeding grouse of growing tips of heather which contain high levels of nitrogen and phosphorus (Moss, J. Anim. Ecol., 41, 411; 1972). Moss, Watson and Parr confirmed that maternal nutrition did affect breeding success but what was important was the length of time during which heather growth was possible prior to laying (J. Anim. Ecol., 44, 233; 1975). Management plans for grouse moors are aimed at creating conditions for maximum breeding success and standing crop of grouse. On wet moors craneflies are not available until after egg laying and so cannot directly influence pre-laying nutrition. Their peak in abundance comes shortly after clutches are completed when, it could be argued, female grouse are in a state of nutrient depletion. By offering an easily gathered, rich food source at this time, they could play a vital part in the maintenance of grouse populations. A full-scale investigation of the relationship between grouse and insects seems now to be overdue, for if these preliminary findings are generally sub-

stantiated the future management of the wet third of Britain's grouse habitat may well reflect the need to maintain suitable cranefly sites.

Tropomyosin in transition

from E. J. O'Brien

IN a resting muscle the level of Ca²⁺ is very low and interaction between the thick, myosin-containing and the thin, actin-containing filaments is inhibited. When the muscle is stimulated, Ca²⁺ is released from the sarcoplasmic reticulum so that its concentration around the filaments rises sharply. The inhibition is then removed and myosin and actin can interact, split ATP and produce contraction. The means by which inhibition and its removal are obtained has been extensively investigated, and, in the main, attention has been directed at the proteins troponin and tropomyosin which are combined with actin in the thin filaments of many types of muscle.

In the electron microscope actin resembles two strings of beads twisted slowly around each other. Much more difficult to see, because of its narrow width (2 nm), is tropomyosin, but from diffraction and other studies of the thin filament, it has been shown to lie end-to-end in each of the two grooves between the actin strings. Attached at intervals of about 40 nm along the actin–tropomyosin complex is troponin, which has three components: one, TN-T, which binds to tropomyosin, one, TN-C which binds Ca²⁺ and one, TN-I, which is responsible for inhibition.

With TN-C removed the thin filament is not Ca²⁺-sensitive and remains permanently 'switched off', as in a resting muscle. Without any of the troponin components the actin–tropomyosin complex is also not Ca²⁺-sensitive, but in this case it stimulates the splitting of ATP by myosin, that is, it is 'switched on'. Wakabayashi, Huxley, Amos and Klug (J. molec. Biol., 93, 477; 1975) have studied the nature of the structural change involved in the on–off transition. Since the required structural detail is at the limit of resolution provided by negatively stained material, their analysis has relied heavily on the use of sophisticated computer processing of electron micrographs. This involves densitometry of the micrographs to produce a digital scan, and from this, and a knowledge of actin's helical symmetry, three-dimensional reconstructions of the filaments can be obtained.

Wakabayashi et al. prepared fila-

ments of actin+tropomyosin and of actin + tropomyosin + TN–T + TN–I and formed them into paracrystals by the addition of Mg^{2+}. From the digital scans of micrographs of the paracrystals they selected one filament at a time and reconstructed it in three dimensions. From many such reconstructions the best averages were taken and the two types of filament compared. In the first type of filament the tropomyosin may be clearly distinguished running near the centre of each of the grooves between actin strands. When the TN–T and TN–I are added, however, the tropomyosin is displaced through about 1 nm and becomes much more closely integrated with each actin strand.

From observations of changes in the X-ray diffraction pattern of living muscle at rest and during contraction, several authors have proposed that tropomyosin moves in the actin grooves when the muscle is stimulated. This leads naturally to the proposal that inhibition is caused by blocking by tropomyosin of the sites of actin that interact with myosin. On stimulation of the muscle, increased Ca^{2+} concentration is thought to cause a conformational change in troponin with a consequent displacement of tropomyosin towards the centre of each actin groove and removal of the inhibition. Since the distribution of troponin is discrete along the thin filament (one troponin for every seven actin subunits), whereas that of tropomyosin is continuous, the function of the latter is both to mediate and to amplify the effect of the former.

Because only the amplitudes and not the phases of reflections in the X-ray diffraction patterns are recorded, the tropomyosin movement model cannot be substantiated from the whole-muscle studies alone. This is where the electron microscope work proves so useful. The direct determination by Wakabayashi *et al.* of a tropomyosin shift under the influence of TN–I is therefore an important advance in understanding thin filament control.

The simplicity of the steric blocking mechanism is appealing, but not necessarily convincing, and it may only be a part of a system which involves other subtler allosteric interactions. It has also still to be demonstrated that in the so-called inhibitory position, the tropomyosin does actually cover the actin site where myosin would attach. There is still work to be done then. Moreover, evidence has recently been accumulating for a Ca^{2+}-sensitive regulatory role in the thick as well as in the thin filaments of vertebrate muscles The full picture is bound to be complex and even the distinction between the process and regulation of contraction may become less clear-cut.

Interferon at Warwick

from Mike Clemens

Interferon seems to be a popular subject this summer. The most recent of several meetings on it was an EMBO Workshop held at the University of Warwick on July 29–31 in which much new information concerning the induction, purification and mechanism of action of this anti-viral protein was presented.

IT is well known that the synthesis of interferon can be induced in cells not only by intact viruses but also by synthetic double-stranded polynucleotides. But it is not at all clear whether the latter have to enter cells or act at the surface membrane. D. Hutchinson (University of Warwick) gave a critical account of the evidence in favour of the latter conclusion. In spite of the almost inevitable solubilisation of some double-stranded RNA which occurs when cells are incubated with this inducer covalently bound to inert supports, direct action at the cell surface seems likely. The immediately subsequent events are still a mystery but considerable progress has been made in identifying the messenger RNA for interferon in cells exposed to double-stranded RNA. P. Pitha (Johns Hopkins University) showed that poly(A)-containing RNA from induced human fibroblasts was translated both in mouse cell-free systems and in intact frog oocytes to give products with human interferon activity. No interferon mRNA could be detected in uninduced cells and it seems that regulation of interferon production may well occur at the transcriptional level. D. Burke (University of Warwick) presented evidence that the inhibitor of transcription camptothecin prevents induction of interferon by virus but not by double-stranded RNA, but it is not yet known whether a virus-directed or a host-directed transcriptional event is involved in this effect.

The most controversial area of interferon research concerns its mechanism of action. This has recently been studied by M. Revel (Yale University) using somatic cell hybrids containing a limited number of human chromosomes. The requirement for chromosome 21 for sensitivity of cells to human interferon is now thought to be because of a specific membrane receptor for interferon coded by this chromosome, rather than an intracellular 'anti-viral protein'. Studies with human fibroblasts monosomic or trisomic for chromosome 21 have confirmed this conclusion.

The events that occur between inter-

action of interferon with its receptor and inhibition of virus replication remain something of a mystery. There was general agreement that protein synthesis is a major target for interferon action but D. Metz (National Institute for Medical Research) produced convincing evidence that, in the case of monkey cells infected with SV40 virus, there is selective inhibition of viral RNA synthesis or enhanced nuclear turnover of viral transcripts after interferon treatment. The recently discovered process of methylation of the 5' ends of newly synthesised mRNA may also be a target for interferon action. P. Lengyel (Yale University) reported that the rate and extent of methylation of reovirus mRNAs in interferon-treated cell extracts was only half that seen in control extracts. However, A. Ball (University of Connecticut) stated that, in an interesting coupled transcription–translation system, methylation of newly synthesised vesicular stomatitis virus RNA is not impaired in the presence of extracts of interferon-treated chick embryo cells. In the same incubations translation of this RNA was severely inhibited compared to its translation in control cell extracts.

A possible clue to the mechanism of inhibition of translation came from the observation by I. M. Kerr's laboratory (NIMR) that interferon-treated L-cell extracts are more sensitive to inhibition by double-stranded RNA than control extracts. T. Hunt (University of Cambridge) described some recent experiments demonstrating that, in reticulocyte cell-free systems, double-stranded RNA causes the ATP-dependent phosphorylation of an initiation factor. However several groups have shown that polypeptide chain elongation as well as initiation is impaired by interferon treatment so that the situation is rather complex. Lengyel and Ball both stated that the established ability of tRNA to reverse the interferon-mediated inhibition of translation is not observed under certain conditions and the physiological relevance of this effect must therefore remain open to doubt. It would be a brave person who predicts, on the basis of present knowledge, just how interferon prevents viral replication within animal cells.

Correction

In the article by Donald Boulter "Breeding for Protein Yield and Quality" (*Nature*, **256**, 168; 1975) there occurred the phrase "the long-assumed negative relationship between protein yield and grain yield". The assumed negative relationship was in fact between protein content and grain yield.

review article

Analogies between embryonic (T/t) antigens and adult major histocompatibility (H-2) antigens

Karen Artzt* & Dorothea Bennett*

Progress has been made in defining cell surface components specified by the T/t locus in the mouse, both in their expression on sperm and, in the one case studied, on normal embryonic cells and teratocarcinoma. Some striking analogies between this putative embryonic cell recognition system and the adult major histocompatibility complex are presented.

IT is now generally accepted that cell surface structures have a critical role in cellular interactions during development and that they are genetically programmed. We are fortunate, therefore, to have available in the mouse an extensive set of mutations at the *T/t* locus that not only control specific points in embryonic development, but apparently operate through the specification of cell surface structures on embryonic cells and sperm. The serological identification and biochemical study of the products of *T/t* locus genes may offer some new insights into the genetic control and mechanism of development.

The *T/t* locus

The *T/t* complex is located on chromosome 17, fourteen crossover units to the left of *H-2*. It is marked by a class of semi-dominant *T* (Brachyury) mutations which, when heterozygous, cause a short tail and when homozygous are lethal[1,2]. But more important, *T* mutations interact with recessive alleles at this locus to produce a tailless (T/t) phenotype; this interaction makes possible the detection of recessive *t* alleles that would otherwise go unnoticed.

A large number of different *t* mutants have been detected; as homozygotes some are embryonic lethals, some are semilethal (some, but not all, homozygotes of this class die), and many are viable. Here we will consider mainly the lethal ones. These lethal mutations are not laboratory curiosities since they exist as natural polymorphisms in wild populations of mice the world over, where they are maintained by a distortion of their rate of transmission through males (but not females). Better than 90%, instead of the expected mendelian 50%, of the progeny of a heterozygous male (+/*t*) and a normal female (+/+) will carry the *t* allele[3]. Lethal *t* mutants also have the unique property of suppressing normal recombination, by some mechanism still obscure, along 14 units of chromosome between *T* and *H-2* (ref. 4, D.B., unpublished data and C. Hammerberg and J. Klein, personal communication). About one gamete in 10⁻³ does, however, carry a recombinant chromosome, as judged by the exchange of an outside marker. This kind of recombination event almost always generates a new and different *t* allele[5], usually viable. Observation of these transitions is facilitated because two different *t* mutants complement one another, so that *tˣ/tʸ* animals survive and are normal in all respects except male fertility (for reviews see refs 3, 6 and 7).

*Address: Department of Anatomy, Cornell University Medical College, New York, New York 10021.

The *T/t* locus clearly contains a set of genes of critical importance in early stages of embryonic development. We know that normal alleles are essential for normal development because homozygotes for any one of eleven different mutant alleles die at characteristic stages during embryogenesis, usually when a particular cell type proves incapable of maintaining itself or proceeding to the next step in differentiation. Morphological studies of affected homozygous embryos[6,8] provide a dramatic picture of failure of cell–cell interactions, and suggest strongly that the initial defect can be associated with the cell membrane[9,10].

These observations led in 1970 to a search for membrane structures specified by the *T/t* locus. Since sperm cells are also affected by *t* mutations[11] and since they were readily obtainable in quantity, whereas young embryos were not, sperm were used initially to define T/t cell surface structures. Immunisation of mice with mutant (*T/t*) sperm and appropriate absorption of the resulting antisera with + sperm yielded specific antibodies that were demonstrated in complement-mediated cytotoxicity tests to be directed against the products of *T* and several different recessive *t* mutations[12,13]. These initial studies and work in progress show: that codominantly specified T/t antigens exist on the sperm surface, that certain of the different *t* haplotypes produce unique specificities whereas others are cross reacting, that cross reacting antigens can in turn be resolved by absorption analysis into multiple specificites[14], and finally, that some of these specificities are separated from one another by the rare recombination events that generate a new *t* allele from a pre-existing one (unpublished results).

Once it was established that mutations in the *T/t* complex were in fact associated with serologically detectable alterations of the sperm surface, the task was to validate our hypothesis that these antigens also appeared on specific groups of embryonic cells at particular stages of development. It is technically difficult to test this assumption using mutant embryos. We therefore turned to the study of teratoma cell lines established and characterised in the laboratory of F. Jacob, to test the more general hypothesis that embryonic cells can be characterised by their display of genetically controlled cell- and stage-specific antigens.

Teratoma system

Teratomas are tumours that develop spontaneously from germ cells, or from experimentally manipulated embryos, and represent a form of disorganised parthenogenesis that gives rise to derivatives of all three germ layers: ectoderm, mesoderm

and endoderm[15]. Male mice of strain 129/Sv produce a high proportion of such tumours, and these tumours have been extensively studied both *in vivo* and *in vitro*[16]. An interesting and technically very helpful feature of teratoma cell culture lines is that different derivatives of the same tumour may form stable lines with different capacities for differentiation. For example, essentially pure cultures of the following types have been isolated. First, lines containing primitive teratocarcinoma (PTC) stem cells that are multipotential and capable of producing derivatives of all three germ layers. Second, lines containing differentiated teratocarcinoma (DTC) cells that are restricted to a particular pathway in development, and capable of producing only a limited array of differentiated cell types, for example yolk-sac carcinoma, or myoblasts. Third, lines containing PTC cells that have lost all capacity for differentiation and are nullipotential. Cultures of the last two kinds provide relatively homogeneous populations of embryonic cells that seem to be permanently trapped at specific developmental steps, and therefore can be used to attempt to identify cell- and stage-specific surface components.

The hypothesis was that cell surface structures concerned with early embryonic development appear and disappear long before immunological self tolerance, and therefore would behave as autoantigens[17]. (T/t antigens are on adult sperm but act as autoantigens to males because of their immunologically privileged location within the seminiferous tubules.)

The most simplified PTC cell line (F9) of the third category above was used to prepare syngeneic hyperimmune antisera against irradiated cells. This antiserum was assayed in complement-mediated cytotoxicity tests, and by absorption; it was found to react only with lines of teratoma which contained PTC cells, of either nullipotential or multipotential variety, but not with differentiated lines. The serum was also negative on all adult somatic cells tested, as well as a wide variety of tumour cells. Importantly, however, the anti-F9 serum reacted with sperm cells and normal cleavage stage embryos[18]; expression is low on one-celled embryos, increases to a maximum at about 8 cells, and then declines again (unpublished data). The exact timing of the disappearance of the antigen from the embryo is still under study, but it is not present on any differentiated cells that have been examined.

These results suggested that anti-F9 recognised a cell surface structure present on uncommitted cells and absent from differentiated cells. The antigen recognised was not a tumour antigen, and its normal tissue distribution was restricted to sperm and early embryos.

Relationship of F9 antigen to *T/t* locus

The general distribution of F9 parallels that postulated for T/t antigens. The parallel was quite precise with respect to a particular *t* lethal (t^{12}) as t^{12} homozygotes become abnormal and die at the morula stage. It seemed a promising possibility that the antigen detected on PTC-F9 cells, normal morulae and sperm, might be specified by the wild type allele of t^{12}, since of course, the genotype of the teratoma did not contain t^{12}, but rather (+) alleles at the *T/t* locus. Tests of this possibility were available, since the expression of T/t antigens on sperm is codominant[13]. Thus, if F9 were specified by the (+) allele of t^{12}, sperm from $+t^{12}$ males should carry only half as much F9 antigen as sperm from $+/+$ males.

The relationship of F9 to $+t^{12}$ was therefore tested by absorbing anti-F9 with known numbers of sperm from males heterozygous for t^{12}, and males that were either wild type or heterozygous for other *T/t* mutations. It was consistently found that sperm from males carrying t^{12} were only half as effective as all other types in removing anti-F9 activity[19]. The relative paucity of F9 antigen on sperm from t^{12} heterozygotes was confirmed with direct cytotoxicity tests of anti-F9 on sperm from various genotypes; T/t^{12} sperm were only half as susceptible to complement dependent lysis as were sperm from $T/+$ males or males carrying different later-acting *t* alleles (T/t^{0}, T/t^{w5}, T/t^{9}) (unpublished results). Thus, F9

antigen seems to be specified by the $+t^{12}$ gene.

To recapitulate, these experiments have been encouraging on three fronts: first, teratoma cells at a defined stage of differentiation have proved to be valid models for cells of the normal embryo; second, a genetically specified, stage-specific, embryonic cell-surface antigen (F9) has been defined; and third, the appearance of this antigen in the embryo has been shown to correlate temporally with the developmental function of the gene responsible for it. The functional role of this antigen in development is as yet unknown, but it may reasonably be speculated that it is concerned with cell–cell interactions.

Analogy between the *T/t* complex and MHC

Our own conceptual framework for some years has been that the *T/t* locus may represent an embryonic analogue of the adult major histocompatibility complex (MHC), and that both systems operate as mediators of cell–cell recognition; the former functioning in the embryo, the latter in the adult. The evidence in support of this hypothesis is as follows.

First, the *T/t* complex and the *H-2* complex of the mouse are linked on chromosome 17. Their linkage may not be entirely fortuitous. In the presence of most lethal and semi-lethal *t* alleles, recombination is suppressed between *t* and *H-2*, a distance of 14 cmorgans. This suppression is unexplained, but led Snell[20] as early as 1968 to propose that the two loci comprised a "super gene" acting as a functional unit for maintaining heterozygosity at *H-2* in feral populations of mice.

Second, each complex occupies a large region of chromosome. The *H-2* complex spans 0.4 cmorgans[21]; the *T* complex is not, as yet, measured because of the recombination suppression associated with *t* alleles; but this fact alone, and evidence that *t* mutation consists of several different functional elements separable by exceptional recombination, suggest that it also occupies a long region.

Third, both *T/t* and *H-2* specify codominant antigens on the cell surface which are complex and highly polymorphic. Each *H-2* haplotype of independent origin encodes on the average eight specificities[22]. Haplotypes of *t* mutants have three or four specificities; the total number currently defined is six, but this number rests on the analysis of only three cross reacting haplotypes[14]. Furthermore, since wild type specificities have not been taken into account at all, the six specificities so far defined probably represent only a fraction of the total.

Fourth, each complex locus has a minimum of two sero-logically detectable (SD) genes. The two SD regions of *H-2* are well documented[23]. The *t* lethals have long been known to consist of at least two functionally different regions separable by crossingover, namely a factor responsible for lethality and a factor responsible for phenotypic interaction with *T*. This is made obvious by the generation of viable alleles by recombination[24].

We have studied several of these recombinant haplotypes serologically, and in every case direct cytotoxicity tests demonstrate cross reactivity with the parental haplotype. In the one case analysed by absorption, when antiserum specific for the parental lethal is thoroughly absorbed with the recombinant *t* viable sperm, it retains activity for the parental lethal and for other cross reactive lethal haplotypes. On the other hand, the absorbed antiserum is negative on other viable haplotypes independently generated from the same parental lethal haplotype. The serological data are consistent with the functional criteria mentioned above and suggest that the crossover haplotype has lost the unique specificities associated with lethality but retained other mutant specificities (unpublished).

Fifth, the developmental expression of H-2 and T/t antigens seem to be reciprocal. H-2 is present on all adult cells, with the apparent exception of sperm[25], but absent from the early embryo[26] and analogous cells such as F9 (refs 25 and 27). T/t, on the other hand, is absent from all adult cells except germ cells[12], but is present on embryonic cells[19]. H-2 is first detectable in 7–9-d embryos[28], whereas T/t antigens associated

with lethal haplotypes are presumed to cease expression by 9 days of development. Furthermore, as *in vitro* populations of multipotential teratoma cells differentiate, detectable levels of F9 antigen fall, while H-2 shows a concomitant rise[29].

Sixth, both systems seem to have analogues in other species. MHC complexes like *H-2* have been found in all mammals studied, as well as birds[21]. Likewise, antigens cross reacting with F9 ($+t^{12}$) have been found on all mammalian sperm examined[30], including human sperm[31,32].

Finally, recent biochemical evidence suggests that the antigenic products of both *T/t* and *H-2* are structurally similar. Lactoperoxidase radioiodination of cell surface components of lymphocytes, sperm and F9 cells, and immunoprecipitation with appropriate antisera, show not only that the molecular weight and subunit structure of the D and K products of *H-2* and the F9 ($+t^{12}$) antigen are identical, but also that $+t^{12}$, like H-2, is associated with a B-2-microglobulin-like moiety in the membrane[25]. Similar results have been reported for the TL antigen[33,34] which is closely linked to *H-2D* and apparently has a reciprocal interaction with it in the plasma membrane. Amino acid sequencing of $+t^{12}$ is now under way and together with sequence data for H-2, will prove or disprove the homology strongly suggested by these data.

The considerations above suggest that the *T/t* complex may be an evolutionary precursor of the MHC, or that both complexes originated in a common ancestoral gene. An embryonic recognition mechanism of the type associated with *T/t* genes could have evolved with the metazoans at the stage when an immune system was not yet organised, indeed, not yet necessary. Later, with the advent of vertebrates and especially warm blooded animals, when protection from microorganisms became imperative, the duplication and specialisation of a complex genetic region already governing cell recognition may have been an economical way of providing genes for immuno-logical recognition.

1 Chesley, P , *J. exp. Zool.*, 70, 429–459 (1935)
2 Bennett, D., *et al* , *Gen. Res* (in the press).
3 Dunn, L C., *Science*, 144, 260–263 (1964)
4 Green, M. C., and Snell, G. D., *Mouse News Lett.*, 40, 29 (1969)
5 Lyon, M F., *Heredity*, 14, 247–252 (1960).
6 Bennett, D., *Science*, 144 263–267 (1964).
7 Bennett, D , *Cell* (in the press).
8 Spiegelman, M , and Bennett, D , *J. Embryol. exp Morph.*, 32 723–728 (1974).
9 Bennett, D , Boyse, E A., Old, L J , in *Proc. third Lepetit Colloquium*, 247–263, (North Holland, Amsterdam, 1971).
10 Artzt, K., and Bennett, D., in *Fogerty International Workshop on Cell Surfaces and Malignancy* (in the press).
11 Bennett, D , and Dunn, L C , in *Proc. Symp. Immunogenetics of the H-2 System*, *Liblice-Prague* 1970, 90–103 (Karger, Basel, 1971)
12 Bennett, D , Goldberg, E., Dunn, L. C , and Boyse, E. A , *Proc. natn Acad. Sci. U S.A* , 69, 2076–2080 (1972).
13 Yanagisawa, K., Bennett, D., Boyse, E. A , and Dimeo, A , *Immunogenetics*, 1, 57–67 (1974).
14 Bennett, D , in *Mammalian Early Development* (edit. by Balls, M., and Wild, A.) (Cambridge University Press, London, in the press).
15 Stevens, L. C , *Adv. Morph.*, 6, 1–28 (1967)
16 Damjanov, I., and Solter, D., *Curr. Top Pathol* , 59, 69–130 (1974).
17 Alexander, P., *Nature*, 235, 137–140, 181 (1972)
18 Artzt, K., *et al.*, *Proc natn Acad. Sci U S.A* , 70, 2988–2992 (1973).
19 Artzt, K., Bennett, D., and Jacob, F , *Proc natn. Acad Sci. U.S.A.*, 71, 811–814 (1974)
20 Snell, G. D , *Folia Biologica (Prague)*, 14, 335–358 (1968).
21 Klein, J , *A. Rev Genet.*, 8, 63–78 (1974).
22 Klein, J , and Shreffler, D. C., *Transplant Rev.*, 6, 3–29 (1971).
23 Shreffler, D C , and David, C. S., *Adv Immun.*, 20, 125–195 (1975)
24 Dunn, L C., Bennett, D., and Beasley, A., *Genetics*, 47, 285–303 (1962)
25 Vitetta, E., Artzt, K., Bennett, D., Boyse, E. A., and Jacob, F., *Proc. natn. Acad. Sci. U S A.* (in the press)
26 Palm, J., Heyner, S , and Brinster, R L., *J. exp. Med* , 133, 1282–1293 (1971)
27 Artzt, K., and Jacob, F , *Transplantation*, 17, 632–634 (1974).
28 Edidin, M., in *Transplantation Antigens* (edit by Kahan, B. D., and Reisfeld, R. A), 75–114 (Academic, New York, 1972).
29 Nicolas, J. F., Dubois, P.. Jakob, H , Gaillard, J , and Jacob, F., *Ann. Microbiol. (Instit Pasteur)*, 126A, 3–22 (1975)
30 Jacob, F., in *Mammalian Early Development* (edit by Balls, M., and Wild, A.) (Cambridge University Press, London, in the press)
31 Buc-Caron, M , Gachelin, G , Hofnung, M., and Jacob, F., *Proc. natn Acad. Sci. U S A.*, 71, 1730–1733 (1974).
32 Fellous, M., Gachelin, G., Buc-Caron, M., Dubois, P., and Jacob, F , *Devl Biol.*, 41, 331–337 (1974).
33 Vitetta, E S., Uhr, J. W., and Boyse, E. A., *J. Immun.*, 114, 252–254 (1975)
34 Ostberg, L., Rask, L , Wigzell, H., and Peterson, P. A., *Nature*, 253, 735–736 (1975).

articles

Genetic control of cell size at cell division in yeast

Paul Nurse

Department of Zoology, West Mains Road, Edinburgh EH9 3JT, UK

A temperature-sensitive mutant strain of the fission yeast Schizosaccharomyces pombe *has been isolated which divides at half the size of the wild type. Study of this strain suggests that there is a cell size control over DNA synthesis and a second control acting on nuclear division.*

pombe has been isolated which divides at half the wild-type size. Study of this strain demonstrates that its cell cycle is also profoundly altered compared with wild type. The timing of DNA synthesis in the cell cycle, the size at which cells undergo DNA synthesis, and the size at which cells undergo nuclear division are all different from wild type growing with the same generation time. These observations may be understood in terms of models of cell cycle control that propose a cell size control over DNA synthesis and a second control acting on nuclear division.

GROWING cells tend to maintain a constant size at division[1-3]. This implies that there is coordination between cellular growth and cell division which results in a division occurring when the cell has grown to a particular size. The mechanism by which this is achieved remains obscure although there are various theoretical models which could account for the phenomenon[4]. A new approach to the problem would be the study of mutant strains in which the normal coordination between cellular growth and cell division is disrupted, resulting in the division of cells at different sizes. Such an approach in a simple eukaryote is described in this report.

A mutant strain of the fission yeast *Schizosaccharomyces*

Cell division cycle

The cell division cycle of fission yeast has been extensively studied[5,6]. Cell division is marked by the formation of a prominent cell plate across the middle of the cell, and separation of the two cells occurs a little later. The cell remains constant in diameter, growing only in length during the cell cycle. Cell length is therefore directly related to cell volume. As in higher eukaryotes the DNA–nuclear cycle can be divided into G1, S, G2 and M periods. The S period is very short and occurs at the beginning of the cell cycle

Table 1 Characteristics of wild type and mutant at 25 °C and 35 °C

Strain	Growth temperature (°C)	Generation time (min)	Cell volume at cell division (μm³)		Cell volume at nuclear division (μm³)		Macromolecular content per cell	
			Mean	s.d.	Mean	s.d.	Protein (pg per cell)	RNA (pg per cell)
Wild-type 972	25	228	129	9.1	121	8.4	12.4	2.54
	35	142	149	6 4	139	9 0	14.1	2.90
Mutant cdc9-50	25	232	109	8.8	98.0	7.9	9.66	2.26
	35	138	72.7	7.8	60.4	8.4	7.16	1.54

Cultures of the two strains were grown in a modified EMM 2 minimal medium[5], with the sodium acetate and sodium dihydrogen ortho-phosphate replaced by 15 mM potassium hydrogen phthallate and 10 mM disodium hydrogen orthophosphate. Photographs of cells grown at 25 °C and 5 h after shift to 35 °C were taken using a .× 16 objective in a Zeiss photomicroscope under dark field optics. The length and diameter of 200 cells with cell plates were measured and their volume calculated. Protein and RNA content per cell, and generation times were determined in exponentially growing cultures as described in ref. 12. Between 5 and 10 determinations were made for each estimate of macromolecular content per cell and in no case did the standard error exceed 4% of the mean. Cells with dividing nuclei were detected by staining with Giemsa[5]. The volume of 200 such cells was calculated after correction for cell shrinkage on fixation and staining

Fig. 1 Cell number. nuclei number, RNA, protein, and cell volume at division of wild-type 972 during the transition from exponential growth at 25 °C to 35 °C. A culture of 972 growing at 25 °C was shifted at 0 min to 35 °C, the culture reaching the higher temperature within 5 min of transfer The time of transfer is marked by the large open triangles. Experimental parameters were determined as described in Table 1. The real values of experimental parameters per ml, equivalent to one unit on the arbitrary log scale, is given in brackets in the symbol key. ■, No. of nuclei ml⁻¹ (1 ×10⁶); ▲, cell number ml⁻¹ (1 ×10⁶) ;○, protein ml⁻¹ (21 9 μg); □, RNA ml⁻¹ (5.46 μg), ●, cell volume at cell division.

simultaneously with cell separation. A long G2 period follows culminating in nuclear division about three-quarters of the way through the cell cycle. There is then a short G1 period ending with the start of the S period of the next cell cycle.

Characterisation of mutant strain

The mutant strain cdc9-50 was isolated from wild-type *S. pombe* (972h⁻ obtained from U. Leupold, Bern) after nitrosoguanidine mutagenesis (P. Nurse and B. Carter, unpublished). When grown at 35 °C, cells of strain cdc9-50 divide at about half the volume of wild-type cells at that temperature (Table 1). The mutant phenotype is temperature-enhanced since at 25 °C cdc9-50 divides at a cell volume only 15% smaller than wild type (Table 1). These differences are also reflected in the RNA and protein content per cell in exponentially growing cultures of the two strains at the two temperatures (Table 1). Thus cdc9-50 divides at both a lower cell colume and a reduced macro-molecular content compared with wild type. In spite of these differences cdc9-50 has a similar generation time to wild type at both 25 °C and 35 °C (Table 1).

The mutant phenotype of reduced cell size at division segregated 2 : 2 in all of 27 asci dissected from a wild-type 975 h⁺ × cdc9-50h⁻ cross (P Thuriaux, personal communication), demonstrating that it is caused by a mutation in a single nuclear gene

Size control over nuclear division

Use was made of the temperature sensitivity of the mutant phenotype to determine the stage in the cell division cycle at which the coupling of cellular growth to cell division takes place. Exponentially growing cultures of cdc9-50 and wild type at 25 °C were shifted to 35 °C. Cell number in the wild-type culture continued to increase for 50 min, stopped for about 30 min, and then gradually increased to the normal growth rate at 35 °C (Fig. 1). During the short plateau in cell numbers, RNA and protein continued to increase, consistent with the increase in cell volume at division that is observed at this time (Fig. 1). The temperature shift temporarily inhibits nuclear division, since for 25 min after the shift there is no further increase in the number of nuclei per ml of culture, and the normal rate of increase at 35 °C is only reached after a further 70 min (Fig. 1). Those cells in which nuclear division is temporarily inhibited are delayed from entering cell division, accounting for the observed short plateau and reduced rate of increase in cell number.

On shifting the culture of cdc9-50 from 25 °C to 35 °C, cell number continued to increase for 50 min and then almost stopped, similar to the behaviour observed in wild

type (Fig 2) Unlike wild type, however, the plateau in cell number was very short (10–15 min) and was followed by a rapid rise rather faster than the usual rate of increase at 35 °C (Fig. 2). This rapid rise in cell number was preceded by a dramatic drop in cell volume at division (Fig. 2). RNA and protein continued to rise as in wild type (Fig. 2) and thus the RNA and protein content per cell dropped to the reduced level observed in exponential culture at 35 °C (Table 1) For 20 min after the temperature shift there was no increase in the number of nuclei per ml of culture showing an inhibition of nuclear division similar to wild type (Fig. 2). Thereafter there was a very rapid rise for a period of 40–50 min (Fig. 2), much faster than the normal rate of increase at 35 °C This suggests that nuclear division is initiated in a large proportion of the population after a period of about 20 min at the higher temperature.

These data can be explained in terms of a size control over the initiation of nuclear division. In wild type at 25 °C the cell grows to a critical size and nuclear division is then initiated. On shift to 35 °C this control is modulated so that the cell grows to a larger size before nuclear division can take place. In cdc9–50 the size control is altered so that at 35 °C nuclear division occurs in cells of a smaller size than wild type On shift from 25 °C to 35 °C nuclear division is briefly inhibited by the same modulation that is observed in wild type. The mutant phenotype, however, rapidly expresses itself by reducing the cell size at which nuclear division is initiated. This results in nuclear division occurring immediately in that proportion of the cell population that were too small for initiation at 25 °C, but which were above the smaller critical size required at 35 °C Thus cells that were too small to divide at 25 °C would be accelerated through the cell division cycle and would divide at the smaller size characteristic of growth at 35 °C.

If cdc9–50 has an altered size control over the initiation of nuclear division, then nuclear division should occur in cells of a smaller size compared with wild type. When grown at 35 °C cells of cdc9–50 containing dividing nuclei were about half the volume of similar cells of wild type (Table 1). At 25 °C cells of cdc9–50 containing dividing nuclei were only about 20% smaller than wild type (Table 1) These relative differences are similar to the differences observed in cell volume at cell division for the two strains at the two temperatures. This would suggest that the size control over nuclear division determines cell size at cell division.

Timing of DNA synthesis during the cell cycle

The DNA content per cell in exponentially growing cultures is slightly reduced in cdc9–50 at 35 °C compared with wild type (Table 2) This suggests that a greater proportion of cells have the unreplicated 1C content of DNA in cdc9–50, and hence DNA synthesis occurs a little later in the cell cycle than in wild type. This was checked by
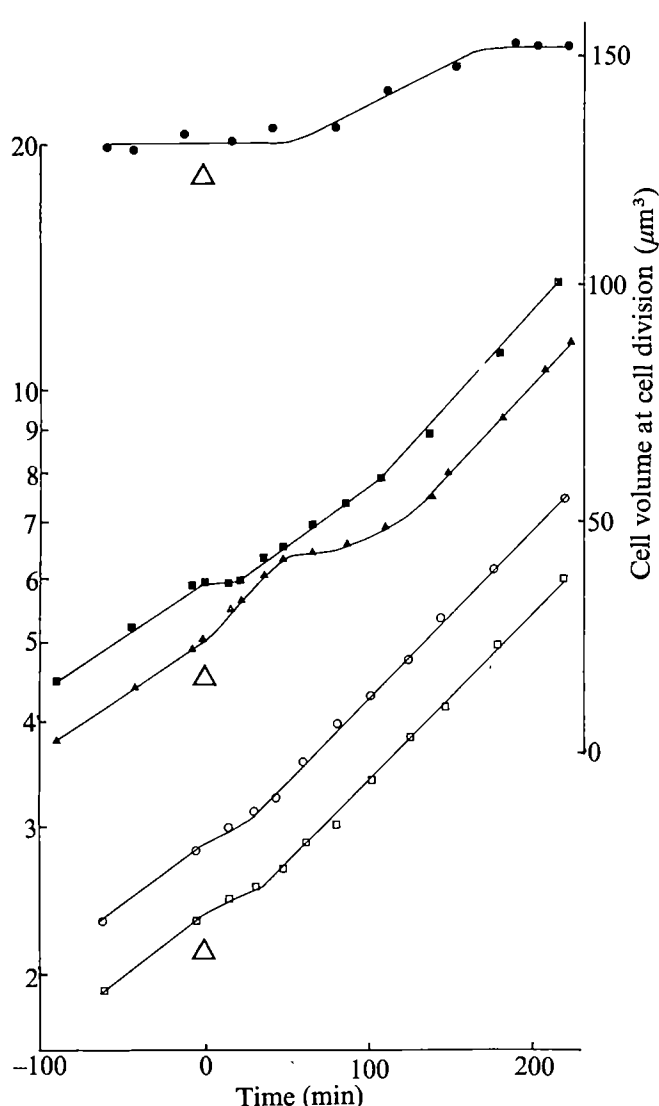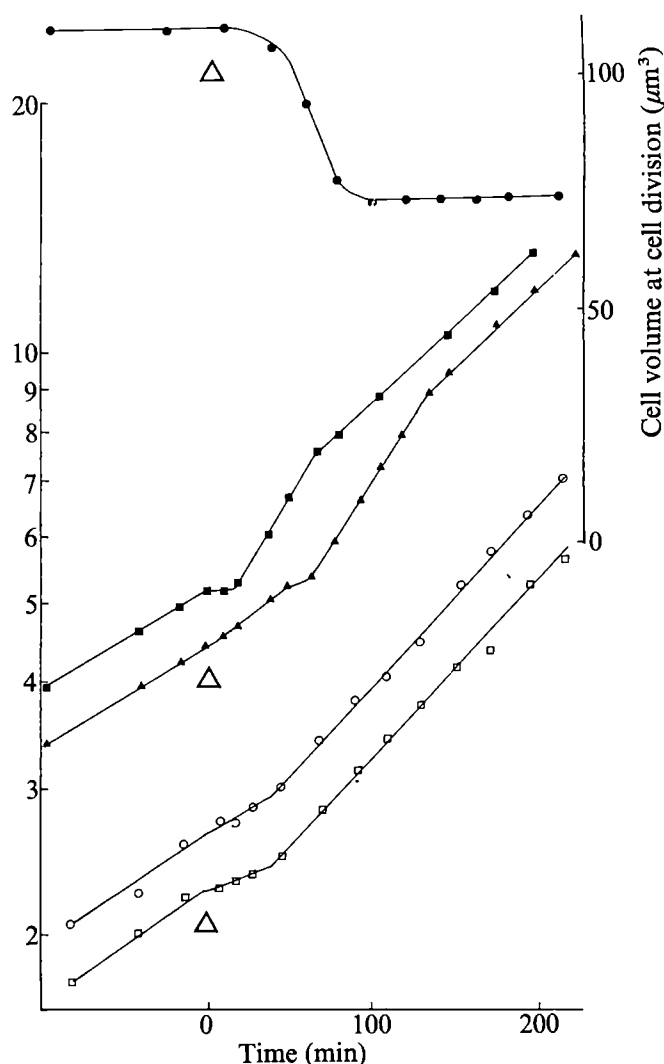

Fig. 2 Cell number, nuclei number, RNA, protein and cell volume at division of cdc9–50 during the transition from exponential growth at 25 °C to 35 °C. All experimental details as for Fig. 1. ■, No. of nuclei ml⁻¹ (1×10^6); ▲, cell number ml⁻¹ (1×10^6); O, protein ml⁻¹ (16 8 µg); □, RNA ml⁻¹ (4.58 µg); ●, cell volume at cell division.

measuring the increase in cell number in exponentially growing cultures after inhibiting DNA synthesis with hydroxyurea[2]. Under these conditions only those cells which have completed DNA synthesis can undergo cell division. The cell number increase for cdc9–50 was reduced compared with wild type (Table 2), again suggesting that DNA synthesis occurs later in the cell cycle in the mutant strain.

Table 2 Timing during the cell cycle and cell size at which DNA synthesis takes place in wild-type 972 and cdc9–50 growing at 35 °C

Strain	DNA content per cell in exponential culture (fg per cell)		Cell number increase after inhibition of DNA synthesis	Fraction of a cell division cycle at which midpoint of rise in DNA occurred	Macromolecular content per cell at midpoint of rise in DNA			
					Calculated		Measured	
	Mean	s e.			Protein (pg per cell)	RNA (pg per cell)	Protein (pg per cell)	RNA (pg per cell)
Wild type 972	34.6	1 4	× 2.04	0.00	10.2	2.09	12.0	2.61
Mutant cdc9–50	28.4	0.77	× 1.86	0.29	6.29	1.35	5 87	1.37

Strains were grown at 35 °C as described in Table 1; DNA, protein and RNA content per cell were determined as described in ref. 12. Inhibition of DNA synthesis was achieved by the addition of 11 mM hydroxyurea. Three synchronous cultures were prepared as described in Fig 3 and the mean fraction of the cell cycle at which the midpoint of rise in DNA occurred was determined (cell separation was taken as 0). The macromolecular content per cell at this time was measured, and was also calculated using the method described in ref. 8 and the data from Table 1.
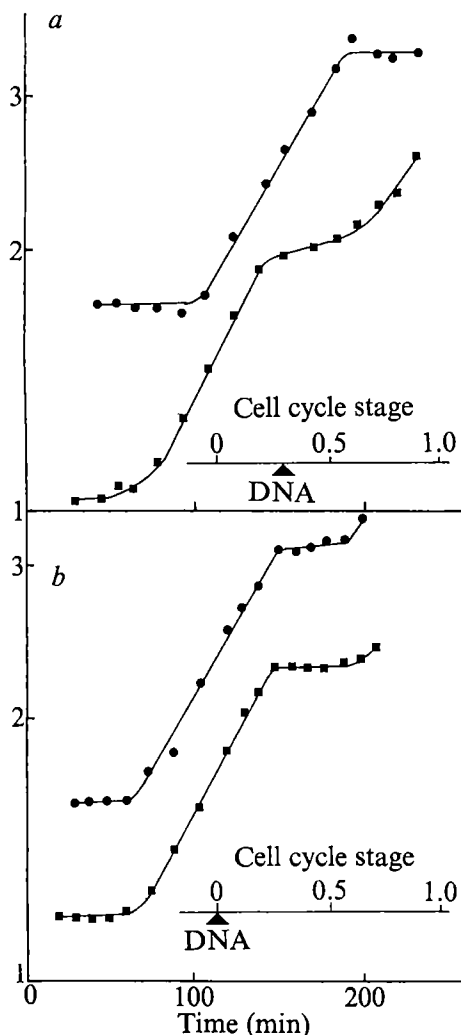
Fig. 3 DNA and cell number during synchronous cultures at 35 °C of *cdc9–50* (*a*) and wild-type 972 (*b*). Strains were grown at 35 °C as described in Table 1. Small cells were selected from these cultures by sedimentation rate centrifugation through a lactose gradient in a zonal rotor[7]. The small cells were used as an innoculum for synchronous cultures and DNA and cell number determined, as described in ref. 12. A cell cycle map is given for both cultures starting at cell separation and showing the time of the midpoint of the rise in DNA. The real values of the experimental parameters per ml, equivalent to one unit on the arbitrary log scale, is given in brackets in the symbol key. *a*, ●, DNA ml⁻¹ (8.63 ng); ■, cell number ml⁻¹ (4×10⁶). *b*, ●, DNA ml⁻¹ (7.47 ng); ■, cell number ml⁻¹ (3×10⁶).

These observations were confirmed by synchronous cultures of *cdc9–50* and wild type at 35 °C. Synchronous cultures were prepared by selection of small cells from an exponentially growing asynchronous population using sedimentation rate centrifugation through a lactose density gradient[5,7]. In wild type the midpoint of the rise in DNA occurred at the beginning of the cell cycle simultaneously with cell separation (Fig. 3, Table 2). This timing is similar to that found in wild type at 32 °C (ref. 6). In *cdc9–50*, however, the midpoint of the rise in DNA occurred at 0.3 of a cell cycle, about 40 min later than cell separation (Fig. 3, Table 2). Thus in *cdc9–50* the G1 period is substantially lengthened at the expense of G2.

Knowledge of the timing of DNA synthesis during the cell cycle, and of the protein or RNA content per cell of an exponentially growing culture enables the protein or RNA content of cells undergoing DNA synthesis to be calculated[8]. Assuming protein and RNA to be accumulated exponentially during the cell cycle[9], the protein and RNA content

of cells undergoing DNA synthesis has been calculated for 972 and *cdc9–50* at 35 °C (Table 2). It can be seen that the macromolecular content of these cells is much reduced in *cdc9–50* compared with wild type. This was confirmed by measuring the actual protein and RNA content of cells at the midpoint of the rise in DNA in the synchronous cultures (Table 2).

These results demonstrate that the mutant strain shows several differences to wild type. The timing of DNA synthesis in the cell cycle, the size at which cells undergo DNA synthesis, and the size at which cells undergo nuclear division are all different from wild type growing under identical conditions with the same generation time. Thus the single genetic lesion in *cdc9–50* has profoundly altered the controls acting on a number of events occurring during the cell division cycle.

Model of cell cycle controls

To explain the pleiotropic behaviour of *cdc9–50* and the maintenance of a constant cell size at division, it is necessary to invoke controls that act at both nuclear division and DNA synthesis. One such model would be that a cell size control acts over the initiation of nuclear division, and a second separate size control acts over an event required for initiation of DNA synthesis. The latter size control refers more precisely to a critical mass per genome which in wild-type cells is attained in the cell cycle previous to that in which DNA synthesis actually occurs. This is because the size control over nuclear division produces daughter cells that are already larger than the threshold size required to initiate DNA synthesis. Since nuclear division is required before DNA synthesis can take place (ref. 10 and my unpublished results using temperature-sensitive mutants defective in nuclear division), DNA synthesis cannot occur early in the previous cell cycle at the threshold cell size, but will be delayed until after nuclear division. Therefore the size control over DNA synthesis would not be expressed. In *cdc9–50* the genetic lesion reduces the cell size at which nuclear division occurs, but does not alter the size control over the initiation event required for DNA synthesis. In this situation the size control over nuclear division produces daughter cells smaller than the threshold size required to initiate DNA synthesis. The cell then has to grow for a further 0.3 of a cell cycle before it is large enough for DNA synthesis to take place. In wild type the size control over DNA synthesis may be expressed only when the cell is growing in adverse conditions. For example, on approaching stationary phase, yeast cells tend to divide at a smaller size[5,11,12], and so the size control over DNA synthesis would be expressed. The operation of the size control would then prevent a new DNA synthesis–nuclear division cycle being started, accounting for the observed accumulation of stationary phase yeast cells in the G1 period of the cell cycle[11,12].

It should be noted that nuclear division could seem to be under a size control if there was a timing mechanism maintaining a constant period between the initiation event required for DNA synthesis and nuclear division. If the genetic lesion in *cdc9–50* resulted in a shortened period compared with wild type, then nuclear division would be initiated earlier when the cell had grown less, and hence cell division would take place at a smaller size. In this situation which is directly analogous to that proposed for the control of the cell cycle in bacteria[13], *cdc9–50* should be considered as altered in a timing mechanism rather than in a cell size sensing mechanism

Thus fission yeast seems to maintain coordination between growth and cell division with cell size controls over DNA synthesis and nuclear division. The latter size control could, however, also be the consequence of a constant timing period between DNA synthesis and nuclear division.

and encouragement, in particular B. L. A. Carter and J. Creanor for advice with the synchronous cultures and P. A. Fantes for first suggesting an altered timing mechanism The work was supported by a grant from the Science Research Council.

1 Schaechter, M , Williamson, J P , Hood, J R , and Koch, A L., *J gen. Microbiol* , 29, 421–434 (1962)
2 Mitchison, J. M , and Creanor, J., *Expl Cell Res.*, 67, 368–374 (1971)
3 Killander, D , and Zetterberg, A., *Expl Cell Res.*, 38, 272–284 (1965), 40, 12–20 (1965).
4 Fantes, P A., Grant, W D., Pritchard, R. H , Sudbery, P. E., and Wheals, A E , *J theor Biol.*, 50, 213–244 (1975)
5 Mitchison, J. M , in *Methods in Cell Physiology* (edit by Prescott, D. M), 4, 131–165 (Academic, New York, 1970)
6 Mitchison, J. M , and Creanor, J., *Expl Cell Res* , 69, 244–247 (1971).
7 Halvorson, H. O., Carter, B L A , and Tauro, P , *Methods in Enzymology*, 21, 462–471 (1970).
8 James, T. W , in *Cell Cycle Controls* (edit. by Padilla, G M., Cameron, I L , and Zimmerman, A), 31–42 (Academic, New York, 1974).
9 Stebbing, N , *J. Cell Sci.*, 9, 701–717 (1971).
10 Culotti, J , and Hartwell, L. H , *Expl Cell Res.*, 67, 389–401 (1971).
11 Hartwell, L H., *Bact. Rev.*, 38, 164–198 (1974).
12 Bostock, C. J , *Expl Cell Res* , 60, 16–26 (1970).
13 Donachie, W. D , Jones, N C , and Teather, R., *Symp. Soc. gen Microbiol.*, 23, 9–44 (1973).

Infective transmission and characterisation of a C-type virus released by cultured human myeloid leukaemia cells

Natalie M. Teich & Robin A. Weiss
Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK

S. Zaki Salahuddin
Litton Bionetics, Inc., Pearl Street, Bethesda, Maryland 20014

Robert E. Gallagher, David H. Gillespie & Robert C. Gallo
Laboratory of Tumor Cell Biology, National Institutes of Health, Bethesda, Maryland 20014

A C-type virus isolated from long term cultures of myeloid cells from a patient with acute myelogenous leukaemia is infectious for a wide variety of cells. The establishment of chronically infected cells enabled us to characterise the virus by biological, immunological, and biochemical tests. The virus is closely related to the simian sarcoma-associated virus isolated from a woolly monkey fibrosarcoma.

THE possibility that C-type RNA tumour viruses are involved in human neoplastic disease has been the subject of much experimentation and speculation recently. In particular, human leukaemia has come under close scrutiny because so many leukaemias from diverse species of animals such as chickens, cats, and mice, have a viral aetiology[1,2]. Although viraemia does not accompany human leukaemia, it is possible that a C-type virus exists in a latent state and some of the well-known 'footprints' of C-type viruses have been detected in a variety of leukaemic tissues. These include: (1) cellular nucleic acid sequences homologous to viral RNA[3-7]; (2) presence of RNA-directed DNA polymerase (reverse transcriptase) activity akin to the viral enzyme[3,8-14]; (3) presence of intracellular proteins that cross react in serological tests with antibodies to 'virus-specific proteins[15,16]; and infrequently (4) electron microscopic identification of virus-like particles[10,12,17,18]. In many of these reports the virus-like components in the fresh human leukaemic cells were shown to be most closely related to similar components from oncogenic primate RNA tumour viruses, especially woolly monkey (simian) sarcoma virus (SSV)[4,5,7,14-16].

Gallagher and Gallo[19] have reported the production and preliminary characterisation of C-type virus particles from long term cultures of myeloid cells from one patient, HL23, with acute myelogenous leukaemia (AML). The uncultured blood cells of this patient contained reverse transcriptase related to that of SSV (ref. 14). The virus released by HL23 cells is called HL23V-1. In addition, they have now reported the release of similar particles from cells derived from a bone marrow specimen from the same patient obtained 14 months after the original blood specimen[20]. This virus is called HL23V-5.

Here we show that these C-type particles are infectious. The virus may be propagated to high titre in several cell types and a quantitative bioassay is described. The production of substantial titres of virus has enabled us to characterise the virus in detail and to compare it with and show that it closely resembles the C-type SSV and its associated virus (SSAV) previously isolated from a fibrosarcoma of a woolly monkey[21].

Infective nature of virus particles

Supernatant fluids from the AML peripheral blood cell cultures containing virus-like reverse transcriptase activity were initially inoculated on to a wide variety of animal and human cells. After transient bursts of viral replication over a period of 3 months, four chronically infected cell lines were established in A204 cells (human rhabdomyosarcoma), WHE2 cells (fibroblastic strain from a whole human embryo), A7573 (canine thymus) cells, and NRK (normal rat kidney) cells. The establishment of chronically infected virus-producing cells was therefore neither a frequent nor a rapid event. A more rapid transmission of the virus to two cell lines (A204; and KNRK, a rat kidney cell line transformed by, but not producing, Kirsten murine sarcoma virus) was obtained, however, within 3 weeks of exposure of these cells to β-propiolactone-inactivated Sendai virus immediately before inoculation with supernatant fluids from the AML cell cultures. Table 1 shows the variety of cells infected with the AML culture fluids and the frequency of infectious transmission to new host cells. The viruses produced by these new host cells are referred to as 'secondary' viruses; the nature of the host producing each secondary virus is indicated in parentheses.

The secondary HL23V-1 viruses will infect a wider variety of cells than the primary virus (Table 2). This may be a result of the virus acquiring a genuinely wider host range or more likely the result of selection by infective transmission of higher titre virus stocks and higher infectivity to particle ratios. Electron microscopy of chronically infected cultures revealed abundant C-type virus particles (Fig. 1). In contrast to the host range of SSAV, most of the HL23V-1 isolates infected 1283 marmoset cells poorly and human cells well (Tables 1–3).

Table 1 Transmission of HL23V-1 infectivity to cells of different species

Cells	Origin	1	2	4	7	10	12
a, Direct infection*		Detection of reverse transcriptase after culture (weeks)†					
A204	Human rhabdomyosarcoma	+	—	—	+	+	+
HT-1	Moloney MSV-transformed hamster cells	—	—	—	—	ND	ND
NC37	Human lymphoblastoid cells	—	—	—	—	—	—
WHE2	Whole human embryo	+	—	—	+	+	+
4178	Squirrel monkey lung	—	—	—	ND	ND	—
1283	Marmoset lung	—	—	—	ND	ND	—
A7573	Dog thymus	—	—	—	+	ND	+
CCL64	Mink lung	—	—	—	ND	ND	—
Tb-1-1u	Bat lung	—	—	—	ND	ND	—
BHK21	Hamster kidney	—	—	—	—	ND	—
NRK	Rat kidney	—	—	+	ND	+	+
b, Sendai-mediated infection‡		1	3	4	7		
A204	Human rhabdomyosarcoma	—	+	+	+		
CCL64	Mink lung	—	—	—	—		
KNRK	Kirsten MSV-transformed rat kidney cells	+	+	+	+		
Q226	Japanese quail embryo	—	—	—	—		

*Human acute myeloid leukaemic (AML) cells were passaged in tissue culture as previously described[19]. At passage 10, when the supernatant had more than a threefold higher incorporation of ^{3}H-TMP in the presence of poly(rA)·oligo(dT) compared with poly(dA)·oligo(dT) in a reverse transcriptase reaction[20], subconfluent cultures of the various cell types were infected with these fluids. 10^5 target cells were seeded in 5-ml flasks containing complete media and polybrene, 2 μg ml⁻¹. After 16 h the cells were washed and treated with 0.5 ml of cell-free supernatant from the AML cell culture (Strain I-1, ref. 20). The cultures were passaged at weekly intervals and supernatant fluids were periodically assayed for reverse transcriptase activity.

†+, ^{3}H-TMP incorporation with poly(rA)·oligo(dT) more than twofold higher than incorporation in response to poly(dA)·oligo(dT); —, less than twofold stimulation. ND, not done.

‡The test cells were exposed to 10^3 haemagglutination units of β-propiolactone-inactivated Sendai virus at 4 °C for 30 min. The cells were then washed and the AML cell culture supernatants were added in 0.2 ml volumes for 60 min at 37 °C; tissue culture medium was then added. Cell cultures were passaged at weekly intervals and assayed as mentioned above.

Quantitative plaque assay for HL23V-1

In addition to screening supernatant samples for reverse transcriptase activity, it is also possible to titrate the HL23V-1 virus isolates by an *in vitro* plaque assay test. The XC syncytial plaque assay technique[22], described and developed for the murine leukaemia viruses, is also suitable for titration of the non-human primate viruses[23], simian sarcoma associated virus (SSAV) and gibbon ape leukaemia virus (GALV)[24]. Utilising the standard technique[22], we have been able to make quantitative determinations of the HL23V-1 virus isolates on various cell types (Table 3). Although the human and rhesus monkey cells show the greatest susceptibility to infection by these viruses, a linear dose–response curve is obtained on all cell lines tested.

The availability of a plaque assay affords the opportunity to study the quantitative neutralisation patterns with sera from normal human subjects, leukaemic patients, and antisera prepared specifically against animal C-type viruses such as SSV. Recently Snyder *et al.*[25] have shown by radioimmunoassays that all human sera contain antibodies to the major proteins and glycoproteins of several C-type viruses, including SSV.

We are currently screening a variety of sera, by plaque neutralisation tests and by a radioimmunoprecipitation assay, for antibodies that cross react with the HL23V-1 isolates. Our tests so far indicate that antibodies that react with HL23V-1 are widely represented in the human population (R. Kurth, T. Oliver, R. W. and N. M. T., unpublished).

Formation and envelope specificity of murine sarcoma virus pseudotypes rescued by HL23V-1

Murine sarcoma virus (MSV) strains are defective for replication and require leukaemia viruses for propagation of infectious stocks. The leukaemia 'helper' virus provides envelope antigens, reverse transcriptase and perhaps other viral structural components for the MSV and the complemented, infectious MSV particles are called pseudotypes. 'Non-producer' cells transformed by MSV can be obtained by infection with high dilutions of MSV, and infectious MSV pseudotypes can be rescued on superinfection of the cells with helper virus. Leukaemia viruses of other species, such as feline leukaemia virus, GALV and SSAV will also rescue MSV pseudotypes on super-infection of susceptible non-producer cells. The KNRK cells

Table 2 Infectivity of secondarily-passaged HL23V-1 viruses as measured by reverse transcriptase activity

Cells	Origin	c.p.m ^{3}H-TMP incorporated per ml per 30 min ($\times 10^{-4}$) after infection with			
		HL23V-1 (A204)	HL23V-1 (KNRK)	HL23V-1 (A7573)	HL23V-1 (WHE2)
A204	Human rhabdomysosarcoma	207	114	188	0.4
HRU-1	Human uterus	96	152	132	1.0
NC37	Human lymphoblasts	18	45	ND	ND
WHE1	Whole human embryo	68	282	168	0.7
WHE3	Whole human embryo	10	80	ND	ND
WI38	Human lung	157	185	ND	ND
CCL64	Mink lung	35	91	48	0.2
Tb-1-1u	Bat lung	397	539	410	0.1
SIRC	Rabbit cornea	198	404	162	12
1283	Marmoset lung	2.3	80	2.6	0 4
NRK	Normal rat kidney	78	53	85	1 1
KNRK	KiMSV-transformed NRK	640	757	71 1	91

Cells of each cell type were seeded at 3.5×10^5 cells per flask (Falcon T30) and infected on the following day in medium containing 20 μg polybrene ml⁻¹. Medium was changed every 2 d and the fluids were collected on day 8 for assay of reverse transcriptase activity. Corrections for background c.p.m. have been subtracted.

used for the propagation of HL23V-1 are transformed, non-producer cells containing the genome of Kirsten-MSV. We therefore assayed the HL23V-1(KNRK) virus for focus-forming units (FFU) on NRK, 7605L, mink, and Japanese quail cells and found titres of up to 10^5 FFU ml^{-1} on all these cell types. The MSV(HL23V-1) pseudotype from infected KNRK cells plated with a linear dose response on NRK cells. The other HL23V-1 isolates, which were not propagated in MSV-containing cells, did not contain focus-forming virus in our assay, but could act as helper viruses. For example, when MSV-containing human cells (kindly provided by Dr P. Peebles) were super-infected with HL23V-1 (A7573), MSV pseudotypes were rescued at a titre of 2×10^4 FFU ml^{-1}.

The MSV pseudotype focus assay can be used as an alternative to the XC syncytial plaque assay in neutralisation tests with antisera. A 1% dilution of antiserum to SSV raised in a goat (kindly provided by Dr F. Deinhardt) which gave a 1,000-fold reduction in titre of MSV(SSAV) neutralised MSV(HL23V-1) to a similar degree. Serum from patient HL23, from whom the virus-producing myeloid cell line was originally derived, did not significantly neutralise MSV(HL23V-1) or MSV(SSAV) pseudotypes. Preliminary tests, however, indicate that weak neutralising activity (approximately 10-fold reduction of FFU using 10^{-1} dilutions of serum) is evident in the sera of some normal individuals and patients in remission stages of myelogenous leukaemia.

The MSV pseudotypes were also used in a virus interference test. 7605L foetal human lung fibroblasts cells were infected with HL23V-1(A7573) or GALV and were challenged after three passages with MSV(HL23V-1) and MSV(SSAV) propagated in KNRK cells. Both leukaemia viruses reduced the plating efficiency of the two MSV pseudotypes to less than 1% that on uninfected 7605L cells. This indicates relatedness among cell receptor sites for SSAV, GALV and HL23V-1. The combined results from neutralisation and interference assays suggest that the envelope proteins of the three groups of viruses are genetically related.

Immunological characterisation of proteins of the HL23V-1 isolates

Biochemical and immunological characterisation of the HL23V-1 virus strains was carried out to evaluate the relationship of the secondary virus isolates to (1) the parental virus isolate, (2) the other secondary virus isolates, and (3) the non-human primate viruses, SSV and GALV.

Antibodies to the reverse transcriptase enzymes from the different C-type viruses have become valuable tools for studying immunological relatedness among these viruses. A quantitative assay for neutralisation of reverse transcriptase activity used antisera prepared specifically against the purified viral enzymes [11,13,19]. We used this technique to analyse the serological specificity of the enzyme activity in supernatant fluids from the myeloid cells producing the primary virus and the cells producing the secondary virus isolates (Table 4).

The viruses produced by the AML cells in culture, HL23V-1

Fig. 1 C-type particles in HL23V-1-infected KNRK cells ($\times 18,600$).

(ref. 19) and HL23V-5 (ref. 20) from peripheral blood and bone marrow, respectively, obtained from the patient several months apart, show nearly identical patterns in these assays of inhibition of reverse transcriptase activity. They are most strongly inhibited by antiserum prepared against the SSV enzyme, less strongly inhibited by anti-GALV enzyme and inhibited least by antiserum to RD114 enzyme (Table 4). This last neutralisation pattern is also seen when purified SSV and GALV enzymes are treated with the RD114 antiserum. By these criteria, the HL23V isolates are most closely related to SSV and GALV.

The specificity of the reverse transcriptases of the secondarily propagated viruses gave much the same pattern as the primary isolates. One interesting anomaly is the HL23V-1 virus grown in A204 cells which may contain two C-type viruses. As with the protein analysis (see below) and the low XC plaque titres, this virus isolate shows a larger cross reaction to RD114 than the other isolates. Also noteworthy is the shift in this pattern with continued passage (compare experiments 1 and 2) which could represent the selection of the SSV-like component over the RD114-like component. Further studies are in progress to elucidate the basis of this phenomenon.

Other tests (not shown) using antisera prepared against the enzymes of feline leukaemia virus, Rauscher murine leukaemia virus, Mason–Pfizer virus, avian myeloblastosis virus, and human cellular polymerase α show little or no inhibition of the HL23V-1 viral reverse transcriptase.

The secondary cells infected by HL23V-1 contained proteins that bound antibodies prepared against p30 protein [26] of SSV and GALV but not with antisera prepared against p30 of mouse or rat leukaemia viruses (R. Gilden, personal communication). In addition, secondarily-infected A204 and A7573 cells contained a protein that reacted with antisera prepared against p30 of baboon endogenous virus and, to a lesser extent, RD114. The p30 proteins of GALV and SSV are not distinguishable by immunological cross-reaction assays [27]; therefore the relatedness of p30 from HL23V-1 to p30 of SSV and GALV indicates that

Table 3 XC syncytial plaque assay of HL23V-1 isolates

Cells	Plaque-forming units per ml of virus				
	HL23V-1 (A204)	HL23V-1 (KNRK)	HL23V-1 (A7573)	HL23V-1 (WHE2)	SSAV
7605L	2.9×10^4	4.8×10^7	7.5×10^5	5.0×10^3	5.0×10^3
WHE2	1.9×10^5	3.0×10^6	5.8×10^5	1.9×10^3	3.0×10^3
FRhL-1	3.6×10^3	2.3×10^6	1.1×10^5	1.0×10^3	3.7×10^5
1283	5.0×10^1	5.5×10^4	5.0×10^2	1.0×10^2	2.0×10^4
NRK	1.5×10^2	7.7×10^5	1.7×10^5	$< 5.0 \times 10^3$	3.5×10^5
SIRC	$< 5.3 \times 10^3$	7.5×10^4	5.5×10^4	$< 5.0 \times 10^3$	5.0×10^3

Cells were seeded at 2×10^5 per 35-mm diameter plates (Linbro) and infected on the following day with 0.1-ml volumes of serially 10-fold diluted virus stocks in the presence of 20 μg ml^{-1} polybrene. Assays were done according to the standard XC plaque method. Reverse transcriptase assays on the various input virus inocula were determined; ^{3}H-TMP incorporated per ml of undiluted virus in a 30 min reaction with poly(rA)·oligo(dT): 6.9×10^6 c.p.m. for HL23V-1 (A204); 1.2×10^7 c.p.m. for HL23V-1 (KNRK); 2.5×10^6 c.p.m. for HL23V-1 (A7573); and 5.8×10^5 c.p.m. for HL23V-1 (WHE2). Cells not mentioned previously: 7605L, foetal human lung fibroblasts (kindly provided by Dr E. H. Macintyre) and FRhL-1, foetal rhesus monkey lung cells (US Division of Biological Standards).

Table 4 Inhibition of reverse transcriptase by antisera to RD114, GALV and SSV

Source of reverse transcriptase	anti-RD114* Expt 1§	anti-RD114* Expt 2§	anti-GALV† Expt 1§	anti-GALV† Expt 2§	anti-SSV† Expt 1§	anti-SSV† Expt 2§
Primary virus-producing cells‡						
HL23V-1	25	33	65	58	78	68
HL23V-5	25	27	47	51	88	52
Secondary virus-producing cells						
HL23V-1 (A204)	60	37	23	54	N.D.	61
HL23V-1 (KNRK)	38	36	64	74	N.D.	83
HL23V-1 (A7573)	40	36	70	53	N.D.	54
HL23V-1 (WHE2)	33	19	68	67	N.D.	77
Immunising enzymes						
RD114	60–70¶		10–25¶		15–25¶	
GALV	10–20		80–90		80–90	
SSV	20–30		80–95		80–95	

% Inhibition of reverse transcriptase activity by

Antisera reactions and reagents are described elsewhere[19]. Inhibition represents the reduction in DNA polymerase activity, using the primer–template poly(rA)·oligo(dT) and ^{3}H-TTP, with an immune serum compared to that with non-immune serum. DNA polymerase activity did not significantly differ when non-immune serum was replaced by 0.1 M Tris, pH 8.0. Values represent the average of duplicate determinations in assays with a minimum of 15,000 c.p.m. above background in the absence of immune serum.

*Antiserum to reverse transcriptase of RD114, an endogenous virus of domestic cats[28-31], which also selectively inhibits the reverse transcriptase of an endogenous primate virus isolated from normal baboon tissues[32,33]. Antiserum kindly provided by Dr R. Gilden.

†Antisera to reverse transcriptases of the two primate C-type viruses. Preparation of antisera in rabbits and rats has been described previously[19].

‡HL23V-1, C-type virus produced by cultured myelogenous leukaemia cells from peripheral blood of patient HL23. HL23V-5, C-type virus from cultured myelogenous leukaemia cells from a bone marrow specimen of the same patient.

§Mean values from determinations achieved with different collections of media in January 1975 (expt 1) and in March 1975 (expt 2) after further passage of the same cultures.

¶Ranges from multiple assays.

all three viruses are members of the same family, not that they are identical.

In a competitive radioimmune assay for p12 viral protein, secondary cells infected by HL23V-1 contain a protein that is indistinguishable from p12 protein of SSV but unrelated to p12 from GALV (Tronick, Stephenson, and Aaronson, personal communication).

Nucleic acid hybridisation

For molecular hybridisation studies using viral RNA, the supernatant fluids of infected cells were clarified, centrifuged through sucrose gradients, and the poly(A)-containing 70S RNA was extracted. All of the HL23V-1 secondary viruses had characteristic 70S RNA and these purified RNAs were then subsequently used for hybridisation reactions.

The viral RNAs were hybridised with cellular DNA of uninfected NRK or KNRK cells or SSV-infected KNRK cells to analyse the genetic relatedness of the HL23V-1 isolates to SSV (Table 5). All the HL23V-1 RNAs show more hybridisation to DNA from those cells infected by SSV than to DNA from uninfected cells. Therefore a qualitative relationship exists

between the nucleic acid of SSV and those of the HL23V-1 isolates. Further studies to determine the degree of homology between the different viral RNAs, by hybridisation between the viral RNAs and their complementary DNA transcripts, are in progress. It is interesting to note the low degree of hybridisation seen between the viral RNAs and DNA from normal human cells; this would indicate that viral genetic material related to HL23V-1 is not maintained as an endogenous genetic component in humans, at least not in all cells.

To elucidate further the genetic relatedness between HL23V-1 and SSV, hybrids formed between SSV 70S RNA and infected and uninfected cellular DNA were subjected to an analysis of thermal stability (Fig. 2). From such profiles, the t_m values (the temperature at which 50% of the hybrid is dissociated) indicate the extent to which the hybrids are well matched in base pairing. The t_m curves are rather broad, indicating heterogeneity in provirus DNA and/or in virus RNA. As can be expected, however, the hybrids formed between SSV RNA and DNA from cells from two different species infected with SSV have a high t_m and therefore better matching of base pair sequences than do the hybrids with normal gibbon ape DNA, cells infected with GALV, or cells infected with one of the HL23V-1 viruses.

Table 5 Genetic relationship between HL23V-1 RNA and SSV provirus DNA

Cell DNA	HL23V-1 (A204)	HL23V-1 (KNRK)	HL23V-1 (A7573)	HL23V-1 (WHE)	SSV (NRK)*	SSV (71AP1)*
NRK	4	25	16	5	20	26
KNRK	7	28	15	13	17	34
KNRK (SSV)†	33	65	48	57	49	49
Normal human	7	3	10	3	5	3
None	2	1	2	0	4	2

% Hybridisation of viral RNAs

Virus-producing cells were labelled for 48 h with 100 µCi ml⁻¹ ^{3}H-uridine. Media was freed of cells by centrifugation at 1,000g for 10 min, adjusted to a final concentration of 1% SDS, 0.1% polyvinylsulphate, and 0.3 M NaCl, and passed over an 0.2 ml bed volume column of oligo-(dT)-cellulose at 25 °C and 1 ml min⁻¹. Poly(A)-containing RNA was eluted with 10 mM Tris, pH 7.4 and centrifuged for 2 h at 22 °C in 5–20% sucrose gradients containing 0.5% SDS, 0.01 M Tris,, pH 7.4, 0.1 M NaCl and 0.001 M EDTA. The 70S RNA was used in hybridisation experiments. Hybridisation mixtures of 0.1 ml consisted of 300 c.p.m. of ^{3}H-RNA (0.3–1 ng), 1 mg of DNA, and 0.3 M PO₄, pH 6.8. The mixtures were flame-sealed in capillary tubes, boiled for 5 min, and then incubated at 60 °C for 7 d (C_0t = 20,000 uncorrected for ionic strength). The contents of the capillary tubes were expelled into 0.8 ml of 2×SSC (0.3 M NaCl, 0.03 M Na citrate, pH 7.0). A 0.2 ml aliquot was precipitated with 10% trichloroacetic acid (TCA); the remaining 0.6 ml aliquot was incubated with 20 µg ml⁻¹ of RNase for 2 h at 37 °C, then precipitated with TCA. The precipitates were collected on nitrocellulose membrane filters, incubated for 1 h at 60 °C with 1 ml of 0.2 M NaOH mixed with 1 ml 50% acetic acid, and counted in 10 ml of Aquasol after incubating 24–36 h at 5 °C in the dark. No TCA-precipitable radioactivity was lost during hybridisation at 60 °C. Percentage RNA hybridised = (c.p.m. in RNase-treated aliquot) (c.p.m. in 0.2 ml aliquot × 3)⁻¹ × 100.

*SSV grown in NRK rat cells or in 71AP1 marmoset cells.

†KNRK cells infected with SSV.
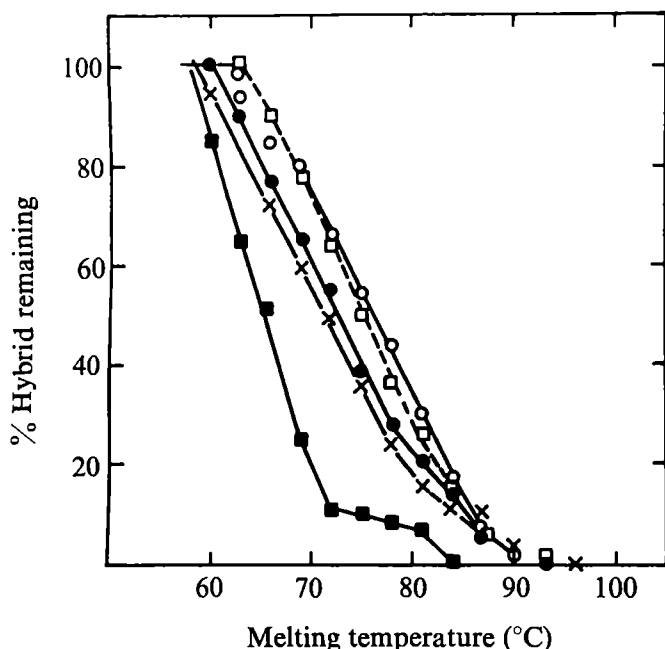
Fig. 2 Thermal stability profiles of hybrids formed between SSV 70S RNA and infected cellular DNAs. [125]I-70S RNA isolated from simian sarcoma virus (SSV) produced by 71AP1 marmoset cells (15,000 c.p.m.; 5×10^7 c.p.m.μg^{-1}) was hybridised at 60 °C in 0.4 M PO_4 to 1,000 μg of DNA isolated from the indicated cells. Hybridisation was carried out to a $C_0 t$ of 20,000 with respect to DNA concentration and uncorrected for (Na⁺). Hybrid solutions were expelled into a 100-fold volume excess of 0.15 M Na⁺, divided into 0.5 ml aliquots, and held at the indicated temperature for 5 min. The solution was brought to final concentrations of 0.45 M Na⁺ and 20 μg ml^{-1} RNase, and the sample was incubated for 2 h at 37 °C. Acid-insoluble material was collected on nitrocellulose membrane filters. ○, HF marmoset cells infected by and producing SSV; □, rat cells infected by and producing SSV; ●, human rhabdomyosarcoma cells infected by and producing HL23V-1; ×, human lymphoblastoid cells infected by and producing gibbon ape leukaemia virus; ■, fresh brain tissue from an apparently normal gibbon ape. The percentage SSV RNA hybridised was 9 % with normal gibbon DNA and 25–50 % with DNA from virus-infected cells.

Although the hybridisation and immunological assays of genetic similarity show that the nucleic acids and proteins of HL23V-1 and SSV are closely related, the t_m experiment suggests that genomic differences do exist between HL23V-1(A204) and SSV. More complete analysis will be presented elsewhere.

Possible implications

It is not surpising to find a C-type virus associated with acute myelogenous leukaemia; viral-related proteins and nucleic acid sequences have been detected in a variety of leukaemias and sarcomas in humans. We have shown here, however, that such virus-related markers can be associated with an infectious virus particle. Freshly isolated leukaemia cells do not release readily detectable C-type virus and the capacity to maintain and propagate the myeloid leukaemic cells in culture for extended periods of time may have allowed production of transmissable virus in this case. Myeloid cell cultures established from an independent, later biopsy of the same patient also release virus particles as measured by reverse transcriptase activity[20]. We have found that this virus, designated HL23V-5, is also infectious for human cells and can be titrated by the XC syncytial plaque assay.

The clear resemblance of the HL23V-1 viruses from human AML cells to the SSAV isolated from a fibrosarcoma of a woolly monkey is striking. The viruses are markedly similar by biological, immunological and molecular criteria. But our data on the relative plating efficiencies on human and 1283 marmoset cells, the inhibition of reverse transcriptase activity

by specific antisera, and the nucleic acid hybridisation studies suggest that, though closely related, the HL23V-1 virus and SSV are not identical. Many precautions were taken to ensure against the possibility of a laboratory contamination[19]; there was no SSAV grown or handled in the biohazard facility in which the myeloid cells or chronically infected cells were maintained. Should we then be surprised by the relatedness of the simian and human viruses? One interesting fact is that the SSV(SSAV) viruses were obtained from a woolly monkey that had been a pet[14] and was therefore in close contact with a human population. We do not know of any C-type viruses isolated from other woolly monkeys. If the HL23V-1 virus and SSAV do indeed have a common origin, it is plausible that the monkey was infected by the human virus. Preliminary serological studies indicate that antibodies to the virus are widespread in the human population. Moreover, a closely related virus has been isolated several times from normal human embryo cells by Panem et al.[34], and Nooter et al. describe elsewhere in this issue the isolation of an SSV-related virus from human leukaemic cells[35]. We should bear in mind, however that isolation of the virus from leukaemic cells does not necessarily mean that the virus is aetiologically related to the leukaemia. Tests are currently in progress to determine whether the virus has oncogenic potential in animals.

Our characterisation of the infectious nature of this virus may be useful in detection and propagation of similar viruses from freshly-isolated leukaemic and solid tumour tissues.

1 Gross, L., *Oncogenic Viruses*, (Pergamon, New York, 1970).
2 Tooze, J , *The Molecular Biology of Tumour Viruses* (Cold Spring Harbor, 1973).
3 Baxt, W , Hehlmann, R., and Spiegelman, S., *Nature new Biol*, 240, 72 (1972).
4 Gallo, R. C., Saxinger, W C., Miller, N , and Gillespie, D., *Proc. natn Acad. Sci. U.S.A.*, 70, 3219–3224 (1973).
5 Miller, N., Saxinger, W. C, Reitz, M. S , Gallagher, R. E , Wu, A. M , Gallo, R. C., and Gillespie, D , *Proc. natn. Acad Sci. U.S.A.*, 71, 3177–3181 (1974).
6 Baxt, W. G., *Proc. natn. Acad. Sci. U.S A.*, 71, 2853–2857 (1974)
7 Mak, T W , Kurtz, S , Manaster, J., and Housman, D , *Proc. natn. Acad. Sci. U.S.A* , 72, 623–627 (1975).
8 Gallo, R. C., Yang, S S , and Ting, R C , *Nature*, 228, 927–929 (1970).
9 Sarngadharan, M. G., Sarin, P. S., Reitz, M. S , and Gallo, R C., *Nature new Biol.*, 240, 67–72 (1972).
10 Kotler, M., Weinberg, E , Haspel, O., Olshevsky, U., and Becker, Y., *Nature new Biol.*, 244, 197–200 (1973).
11 Todaro, G. J., and Gallo, R. C., *Nature*, 244, 206–209 (1973).
12 Mak, T. W , Manaster, J , Howatson, A. F., McCulloch, E. A., and Till, J E., *Proc natn. Acad. Sci. U.S.A.*, 71, 4336–4340 (1974).
13 Gallagher, R. E., Todaro, G. J., Smith, R G., Livingston, D. M., and Gallo, R. C., *Proc natn. Acad Sci. U.S.A.*, 71, 1309–1313 (1974).
14 Mondal, H., Gallagher, R. E., and Gallo, R. C., *Proc. natn. Acad Sci U.S.A.*, 72, 1194–1198 (1975).
15 Sherr, C. J., and Todaro, G. J., *Science*, 187, 855–857 (1975); *Proc. natn. Acad Sci. U.S.A.*, 71, 4703–4707 (1974).
16 Strand, M , and August, J. T., *J. Virol.*, 14, 1584–1596 (1974).
17 Dmochowski, L., *Proc. 14th Ann. Clin. Conf on Leukemia and Lymphoma*, 32–57 (Year Book of Med. Publishers, Chicago, 1970)
18 Cawley, J. C., and Karpas, A., *Eur. J. Cancer*, 10, 559–562 (1974)
19 Gallagher, R E , and Gallo, R. C., *Science*, 187, 350–353 (1975).
20 Gallagher, R. E , Salahuddin, S. Z., Hall, W. T , McCredie, K. B , and Gallo, R C., *Proc natn Acad. Sci. U S A.* (in the press).
21 Theilen, G. H , Gould, D., Fowler, M., and Dungworth, D. L , *J. natn. Cancer Inst.*, 47, 881–889 (1971).
22 Rowe, W. P , Pugh, W. E , and Hartley, J. W., *Virology*, 42, 1136–1139 (1970).
23 Rangan, S. R. S , Ueberhorst, P. J , and Wong, M. C., *Proc. Soc. exp. Biol. Med.*, 142, 1077–1082 (1973).
24 Kawakami, T. G., Huff, S. D., Buckley, P M., Dungworth, D. L., Snyder, S P., and Gilden, R. V., *Nature new Biol* , 235, 170–171 (1972)
25 Snyder, H W. jun., Pincus, T , and Fleissner, E , *Virology* (in the press).
26 August, J. T., Bolognesi, D. P., Fleissner, E., Gilden, R. V., and Nowinski, R. C., *Virology*, 60, 595–601 (1974).
27 Gilden, R. V., Frank, K., Hanson, M., Bladen, S., Toni, R., and Oroszlan, S., *Intervirology*, 2, 360–365 (1973/4).
28 McAllister, R. M., Nicolson, M. O , Gardner, M. B., Rongey, R W., Rasheed, S , Sarma, P. S , Huebner, R. J., Hatanaka, M., Oroszlan, S., Gilden, R. V , Kabigting, A , and Vernon, L , *Nature new Biol.*, 235, 3–6 (1972).
29 Gillespie, D., Gillespie, S., Gallo, R. C., East, J. L., and Dmochowski, L., *Nature new Biol.*, 244, 51–54 (1973).
30 Okabe, H., Gilden, R , and Hatanaka, M., *Nature new Biol.*, 244, 54–56 (1973).
31 Neiman, P., *Nature new Biol.*, 244, 62–64 (1973)
32 Kalter, S S., Helmke, R. J , Panigel, M., Heberling, R. L., Felsburg, P. J., and Axelrod, L. R , *Science*, 179, 1332–1333 (1973)
33 Sherr, C. J , Lieber, M M , Benveniste, R. E., and Todaro, G. J , *Virology*, 58, 492–503 (1974).
34 Panem, S., Prochownik, E. V., Reale, F. R., and Kirsten, W. H., *Science* (in the press).
35 Nooter, K., Aarssen, A. M , Bentvelzen, P., de Groot, F G. and van Pelt, F. G. *Nature*, 256, 595–597 (1975)

Multiple steps in DNA recognition by restriction endonuclease from *E. coli* K

Robert Yuan, Thomas A. Bickle, Walter Ebbers & Christine Brack

Department of Microbiology, Biozentrum of the University of Basel, Basel, Switzerland.

The process of DNA recognition by the activated form of the restriction endonuclease from E. coli *K involves three enzyme–DNA complexes which can be differentiated experimentally. These are: an initial complex formed at a nonspecific site; a recognition complex involving the host specificity site; and a cleavage complex dependent on the presence of ATP.*

THE restriction endonuclease from *Escherichia coli* K, *Eco*RK, is a complex multifunctional protein which cleaves unmodified DNA in the presence of S-adenosylmethionine (Ado–Met), ATP and Mg^{2+} (ref. 1). A powerful ATPase activity appears during the course of this restriction reaction[2] and the same protein is also able to methylate unmodified DNA, rendering it insensitive to its own restriction activity[3].

The way in which the enzyme recognises the host specificity sequence on the DNA, and the factors that determine whether it will act as an endonuclease, or as a methylase, are two aspects of the mechanism which go beyond the immediate area of restriction and modification. The first of these, recognition of nucleotide sequences, is common to most proteins that react with nucleic acids. The second is that the interaction involved in the recognition of the nucleotide sequence directs the protein towards either of two opposed modes of action, restriction or modification. In other words, a specific nucleotide sequence may not only be recognised by a protein, but also act as a signal for the mode of action of a multifunctional enzyme.

In a certain perverse way, it is the very complexity of this protein and the reactions that it carries out that enables us to distinguish experimentally four major stages in the *in vitro* reaction (Fig. 1). These are: Enzyme activation: *Eco*RK binds Ado–Met rapidly, and in a slow second step, an allosteric transition to an activated form (*Eco*RK*) takes place. DNA recognition: *Eco*RK* interacts with DNA, first, at a nonspecific site (initial complex), and then at the unmodified recognition site (recognition complex). The recognition site is the host specificity site on the DNA that confers susceptibility to a given restriction and modification system (the sites for the K system are called sK). Enzyme action: in the absence of methyl groups at the recognition site, the DNA will be either cleaved or methylated. Methylation occurs at the recognition site while DNA scission takes place elsewhere. Loss of nuclease activity: the enzyme is altered following DNA cleavage, and is unable to cleave again. This phenomenon is probably related to the appearance of a vigorous and sustained ATP hydrolysis which begins at the time of DNA cleavage, but continues long after the DNA digest has reached its limit.

This paper deals with the process of DNA recognition. *Eco*RK* can form three distinct complexes with DNA in its restriction mode. It first forms an initial complex at a nonspecific site which is transformed to a more stable recognition complex if an unmodified host specificity site is present on the DNA. Neither of these complexes can be trapped on membrane filters. If ATP is added to the recognition complex, a rapid transition to a third complex which can be trapped on filters, takes place. This presumably occurs at the cleavage site.

Fig. 1 Reaction mechanism of endonuclease *Eco*RK.
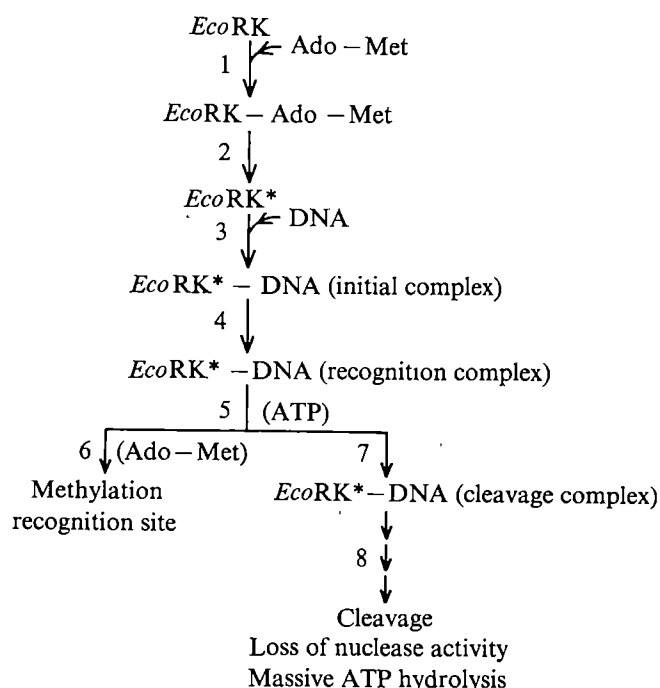
Enzyme forms a specific complex with unmodified DNA that is retained on filters

*Eco*RK has previously been shown to form a specific complex with unmodified λ.0 DNA that can be stabilised with EDTA and detected by binding to nitrocellulose filters. (λ bacteriophage grown on a non-modifying host is designated λ.0; phage grown on a host that confers K-specific modification to the DNA is called λ.K.) The formation of this complex requires the presence of Ado–Met, ATP and Mg^{2+} (ref. 4). No filter-binding complex can be detected with modified λ.K DNA or with DNA that contains no recognition sites for the enzyme. Complex formation reaches its maximum after 2 min at 30 °C, and then decays at a rate consistent with its dissociation after DNA cleavage.

That this complex was not the result of binding to sK sites on the DNA was suggested by experiments with a purified restriction endonuclease from a mutant strain of *E. coli* K12 (ref. 5). This strain is unable to restrict, but can modify normally. As expected, the restriction enzyme purified from it can methylate (but cannot cleave) unmodified DNA *in vitro*. It does not form the filter binding complex. Therefore, *Eco*RK can recognise and methylate λ.0 DNA without forming a filter binding complex. The conclusion then is that the filter-binding complex must be associated with the cleavage reaction, and not DNA recognition proper.

Activated enzyme is stabilised by interaction with DNA

EcoRK* forms two different types of complex with DNA: a nonspecific initial complex and a specific recognition complex. These two complexes can be readily differentiated by observing the effect of λ.K, λ.0 and mutant λ DNAs on the stability of EcoRK*.

The slow allosteric activation to EcoRK* follows the rapid binding of Ado-Met to EcoRK[6]. On dilution to remove free Ado-Met, EcoRK* decays with a half life of 130 s. The effect of DNA on the stability of EcoRK* was determined by adding modified λ.K or λsK$_1$[0]sK$_2$[0].0 DNAs to the dilution buffer. The latter DNA is from a λ–Φ80 hybrid in which all of the sK sites have been eliminated[7]. Clearly both λ.K and λsK$_1$[0]sK$_2$[0].0 DNAs stabilise EcoRK* (Fig. 2a) and thus interact with it, although neither of them is a substrate for the enzyme. Similar stabilisation was observed with λ.0 and T4 DNAs. We define this kind of interaction as initial complex formation.

To detect differences in the interaction between EcoRK* and the various DNAs, the enzyme was activated in the presence of λ.0,λ.K or λsK$_1$[0]sK$_2$[0].0 DNAs and the stability of the complexes formed in these conditions was measured over a period of 60 min following dilution into prewarmed buffer (compared with 6 min in the previous experiment). Figure 2b shows that, although all of the DNAs protect EcoRK* from decay, substrate λ.0 DNA is more efficient than the others. The half life of the activated enzyme goes from 130 s for the enzyme alone to 6 min in the presence of the non-substrate λ.K and λsK$_1$[0]sK$_2$[0].0 DNAs and 22.5 min with substrate λ.0 DNA. This enables us to postulate an initial complex formed by EcoRK* with any DNA, and a more stable recognition complex formed with a DNA that contains the unmodified sK sequence.

Recognition complex is a precursor of the filter-binding complex

The complex formed by activating the enzyme in the presence of λ.0 DNA (that is, the recognition complex) does not bind to nitrocellulose filters, but the addition of ATP converts it to the filter-binding form. The rate at which this occurs seems to follow first-order kinetics with a half time for the reaction of 6.5 s. This is more than 10 times faster than the result obtained when EcoRK* is added to λ.0 ^{32}P-DNA and ATP (Fig. 3a), also a first-order reaction with a half time of about 80 s. Given these rapid binding kinetics, it cannot be ruled out that the recognition complex is, in fact, formed at the cleavage site after interaction with the sK site. Also in this figure are the kinetics of filter binding complex formation when a standard assay was initiated by the addition of non-activated enzyme, and when EcoRK* was diluted into λ.0 ^{32}P-DNA, and ATP was added 2 min later. In the latter case, no DNA binding was observed. Therefore, the rate at which the recognition complex was formed could not be measured directly.

The rate of activation of the enzyme in the presence of un-modified DNA is first or pseudo-first order (Fig. 3b) and is faster than activation of the enzyme alone[6] (respective half lives of 36s as against 54s). Therefore, interaction of the enzyme with DNA displaces the equilibrium towards complex formation and prevents the decay of EcoRK*.

The reaction of the recognition complex with ATP should be second order; therefore some subsequent rate-limiting change in the structure of the enzyme-DNA complex which generates the filter-binding form may explain the apparent first-order kinetics. The recognition complex formed in the absence of ATP was shown to be an intermediate in the restriction pathway. On dilution into ATP, the DNA was rapidly cleaved. The initial complex formed between EcoRK* and λ.K DNA (on dilution into λ.0 ^{32}P-DNA and ATP) produced filter-binding kinetics that were faster than the standard reaction rate and similar to
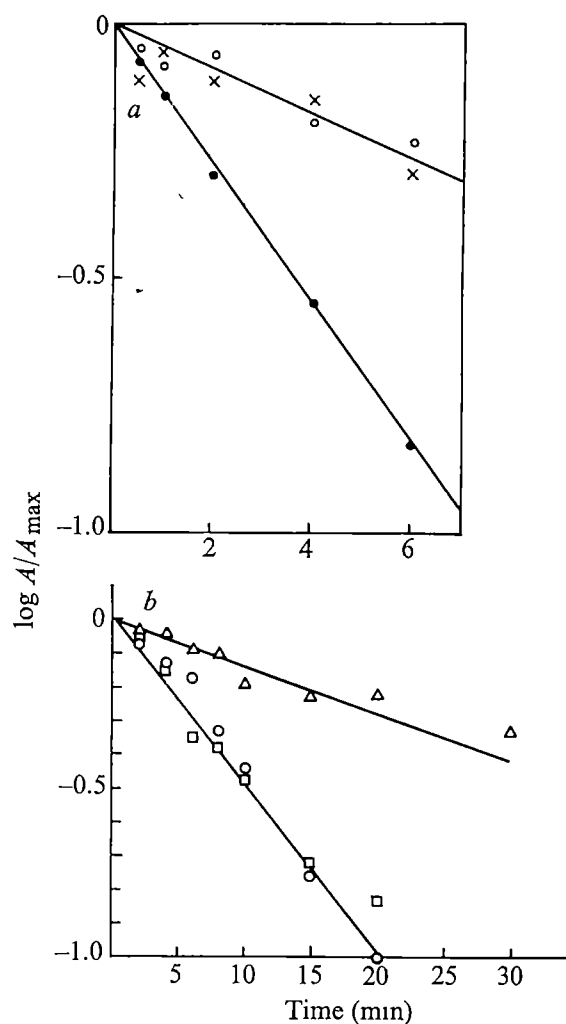
Fig. 2 Effect of DNA on the stability of EcoRK*. a, Stability of EcoRK* in presence or absence of DNA. b, Comparison of effect of different DNAs. a, λ.K and λ.0 ^{32}P-DNA were prepared as described previously[1]. λsK$_1$[0]sK$_2$[0].0 DNA was prepared from phage after heat induction of 803 (h[80]attλsK$_2$[0]cI$_{857}$S$_7$sK$_1$[0]), obtained from Dr N. Murray. EcoRK was purified to homogeneity after modifying the procedure published elsewhere[1]. All reaction mixtures for this and subsequent experiments contained 6 mM MgCl$_2$, 0.26 mM EDTA, 12 mM 2-mercaptoethanol and 100 mM either Tris–HCl or HEPES–OH, pH 8.0. Other additions are detailed in Figure legends. An aliquot (70 µl) of reaction mixture containing 0.13 nmol Ado-Met was mixed with 50 µl (50 µg) of EcoRK, incubated for 5 min at 30 °C, chilled and kept on ice. Aliquots (3 µl) were then diluted into 300 µl pre-warmed reaction buffer containing either no DNA, 0.3 µg of λ.K DNA, or 0.3 µg λsK$_1$[0]sK$_2$[0].0 DNA. After incubation for varying periods, 5 µl of the mixture containing 80 nmol ATP and 0.1 µg λ.0 ^{32}P-DNA (16,700 c.p.m.) were added and the incubation continued for a further 2 min. The reaction was terminated by the addition of 50 µl 0.5 M EDTA and filtered through a nitrocellulose filter. The filters were washed, dried and counted as described previously[4]. b, Three reaction mixtures of 100 µl containing 0.13 nmol Ado-Met each and either 2.4 µg λ.0 ^{32}P-DNA (380,000 c.p.m.), 2 2 µg λ.K DNA or 2.4 µg λsK$_1$[0]sK$_2$[0].0 DNA were prepared. They were incubated at 30 °C for 2 min with 30 µl (6 µg) EcoRK and then rapidly diluted with 11.5 ml prewarmed reaction buffer. Aliquots (500 µl) were removed at various intervals and mixed with either 20 µl ATP mixture (incubation containing λ.0 ^{32}P-DNA) or 20 µl ATP–DNA mixture (the other two incubations). ATP mixture contained 0 2 nmol ATP per 20 µl and ATP–DNA contained 0.2 nmol ATP and 96 ng λ.0[32]P -DNA per 20 µl (15,200 c.p.m.). After addition of the ATP mixture, further incubation was for 1 min, whereas after adding the DNA–ATP mixture the incubation was for 2 min. All reactions were terminated by the addition of 0.5 ml 0.1 M EDTA, filtered and counted as above ●, No DNA; △, λ.0 DNA; ×,λ.K DNA; ○, λsK$_1$[0]sK$_2$[0].0 DNA.

the rate when EcoRK* is added to λ.0 ³²P-DNA and ATP. Dissociation of the enzyme from such initial complexes must therefore be a relatively rapid process and the activated enzyme must interact with many different DNA molecules during protection by non-substrate DNA (compare the half life for protection of 6.5 min with the rapid release detected above).

Recognition complex is insensitive to heparin

The polyanionic glycan, heparin, is an inhibitor of both the DNA binding and cleavage reactions (R. Y., T. A. B., W. E., and C. B., unpublished). This inhibition has been used to study the interactions between the enzyme and substrate DNA. Table 1 shows the results of one such series of experiments. The addition of

Fig. 3 Kinetics of enzyme–DNA interactions. *a*, Kinetics of DNA binding to filters after ATP addition. *b*, Kinetics of enzyme activation in presence of DNA. *a*, Standard reaction kinetics were followed by adding 9 μl EcoRK (1 8 μg) to a prewarmed reaction mixture containing in 4.5 ml: 1.6 μg λ.0 ³²P-DNA (2.16 × 10⁵ c.p.m.), 1.8 μmol ATP and 120 nmol Ado–Met. Aliquots (250 μl) were removed into 25 μl 0 5 M EDTA after different times of incubation at 30 °C and the samples were then assayed on filters. EcoRK* was formed by adding 18 μl EcoRK (3 6 μg) to 190 μl reaction mixture containing 1 nmol Ado–Met and incubating for 3 min at 30 °C. This was chilled in ice and 5 μl aliquots were added to 250 μl reaction mixture containing 0.09 μg λ.0 ³²P-DNA (1.2 × 10⁴ c.p m) and 0 1 μmol ATP. Incubation was carried out for varying time intervals, the reaction was stopped with EDTA and the samples were filtered. In a separate set of incubations, 5 μl of the same EcoRK* was added to 250 μl of a similar mixture, but without ATP, the incubation continued for 3 min and 0.1 μmol ATP was then added After further incubation at 30 °C for varying time intervals, the reactions were terminated with EDTA and filtered. In the last set of reactions, the EcoRK*–λ.0 DNA recognition complex was formed by adding 9 μl (1.8 μg) EcoRK to 90 μl reaction mixture containing 2.4 nmol Ado–Met and 1.6 μg λ.0 ³²P-DNA (2 16 × 10⁵ c.p.m.) and incubating for 3 min at 30 °C. The mixture was chilled in ice and 5 μl aliquots were added to 250 μl reaction mixture containing 0.1 μmol ATP. The samples were incubated for different time intervals at 30 °C, stopped with EDTA and filtered. *b*, Reaction mixture (60 μl) containing 2.75 μg λ.0 ³²P-DNA (2.2 × 10⁵ c.p.m.), 0.13 nmol Ado–Met and 1 μl (0.2 μg) EcoRK was incubated at 30 °C, and at different times 3 μl aliquots were removed and mixed with 200 μl prewarmed buffer containing 0.1 μmol ATP. Each sample was then incubated for a further 2 min, 50 μl 0.5 M EDTA was added and the samples were filtered and counted as before. ●, Standard reaction kinetics; △, kinetics with EcoRK*; ×, incubation of EcoRK* with DNA in absence of ATP; ○, kinetics of filter binding with preformed recognition complex.

Fig. 4 EcoRK*–DNA complexes on glycerol gradients. *a*, Composite showing the positions of EcoRK* and λ DNA. *b*, EcoRK*–λ.0 DNA complex. *c*, EcoRK*–λ.K DNA complex. Four reaction mixtures of 100 μl containing: *a*, 0.45 μg λ.0 ³²P-DNA (63,000 c p.m) *a*, 6.7 nmol Ado–Met and 5 μl (1 μg) EcoRK; *b*, 0 45 μg λ.0 ³²P-DNA (63,000 c p.m.), 6.7 nmol Ado–Met and 5 μl (1 μg) EcoRK; *c*, 0.57 μg λ.K ³H-DNA (132,000 c.p.m), 6.7 nmol Ado–Met and 5 μl (1 μg) EcoRK All reactions were incubated at 30 °C for 3 min, chilled in ice and layered on 4 ml 10–30 % glycerol gradients containing 20 mM potassium phosphate, pH 7.0, 5 mM 2-mercaptoethanol and 0.2 mM EDTA. These were centrifuged at 55,000 r.p.m. for 100 min at 4 °C in an international SB 405 rotor. Twenty fractions were collected from each gradient and 50 μl aliquots were removed from each fraction from *a*, *b* and *c* for counting. Aliquots (50 μl) of *a′* and *c* were assayed by the standard procedure except that Ado–Met was omitted from the incubation Aliquots from *b* were assayed similarly except that both Ado–Met and additional λ.0 ³²P-DNA were omitted.

Table 1 Inhibition by heparin of DNA binding to filters by EcoRK

First incubation	Second incubation	λ.0 ³²P-DNA bound (c.p.m.)	% Maximum
Set 1			
1 Complete	—	6,565	100
		5,569	84.8
2 Complete+heparin	—	80	1.2
		70	1.1
3 Complete−enzyme	—	46	0.7
		55	0.8
Set 2			
4 EcoRK+Ado–Met	λ.0 DNA+ATP	2,185	86
		2,541	100
5 EcoRK+Ado–Met +heparin	λ.0 DNA+ATP	46	1.8
		35	1.4
6 EcoRK+Ado–Met	λ.0 DNA+ATP+heparin	35	1.4
		77	3.0
Set 3			
7 EcoRK+Ado–Met+λ.0 DNA	ATP	1,682	87.9
		1,913	100
8 EcoRK+Ado–Met+λ.0 DNA +heparin	ATP	36	1.9
		24	1.3
9 EcoRK+Ado–Met+λ.0 DNA	ATP+heparin	800	41.8
		1,131	59.1

Set 1, Each reaction mixture of 250 μl contained 0.13 μg λ.0 ³²P-DNA (14,650 c.p.m.), 6 7 nmol Ado–Met and 0.1 μmol ATP. Sample 2 also contained 0.25 μg heparin. EcoRK (0.1 μg, 0.5 μl) was added to the samples indicated and incubation at 30 °C was carried out for 2 min. The reaction was stopped by the addition of 50 μl 0.5 M EDTA and filtered.

Set 2, Each reaction mixture of 5 μl contains 27 pmol Ado–Met. In sample 5, 0.25 μg heparin was also present. EcoRK (0.5 μl, 0.15 μg) was added, the samples were incubated at 30 °C for 3 min and diluted with 250 μl of a reaction mixture containing 0.1 μmol ATP and 0.13 μg λ.0 ³²P-DNA (14,650 c p.m.) per 250 μl or a similar mixture containing in addition 0.25 μg heparin. After a further 2 min incubation the reaction was stopped with EDTA and the samples filtered.

Set 3, Procedure as for set 2 except that the λ.0 ³²P-DNA was incubated with the 5 μl enzyme activation mixture rather than added later during the dilution step.

heparin to a standard reaction mixture abolishes the binding of DNA to filters (Table 1, set 1). Heparin also inhibits the activated enzyme, both when it is added to the activation mixture, and when it is added to a dilution buffer containing DNA and ATP (Table 1, set 2). If, however, the enzyme is activated in the presence of λ.0 DNA and then diluted into buffer containing ATP and heparin, little inhibition is observed (Table 1, set 3). If heparin is added to a recognition complex, and ATP is added later, the same lack of inhibition is observed. Therefore, once the recognition complex has formed, the enzyme is no longer sensitive to inhibition by heparin. Normal DNA cleavage is observed when ATP and heparin are added to such a complex.

Both the initial and recognition complexes can be isolated on glycerol gradients

Direct evidence for the various enzyme–DNA complexes was obtained by glycerol gradient centrifugation. Sedimentation through glycerol gradients readily resolves DNA from the free enzyme (Fig. 4a, a composite of two different gradients). To detect the recognition complex, EcoRK was incubated with λ.0³²P-DNA, Ado–Met and Mg²⁺ at 30 °C, chilled and sedimented through a glycerol gradient. Aliquots from each fraction were counted to determine the position of the DNA and further aliquots were assayed for the recognition complex (Fig. 4b). To detect the initial complex, the experiment was repeated with λ.K ³HDNA in place of λ.0 DNA, and the gradient was assayed after addition of ATP and λ.0 ³²P-DNA to an aliquot from each fraction (Fig. 4c). In both cases, the activated enzyme comigrated with the DNA. Surprisingly, a control incubation of enzyme with λ.0 ³²P-DNA showed that the endonuclease was also bound to the DNA in spite of the fact that it had not been activated with Ado–Met (data not shown). This particular complex, however, does not seem to be an intermediate in the restriction reaction as the binding kinetics of this complex after ATP and Ado–Met addition are those of a standard reaction initiated by addition of EcoRK to λ.0 DNA and cofactors. As in the latter case, the reaction is inhibited by heparin.

An enzyme mechanism

Steps 1 and 2 of the reaction mechanism (Fig. 1) have been described in detail elsewhere[6], and involve the fast binding of Ado–Met by the enzyme followed by a slow transition to an activated form.

Here, we are primarily concerned with the phenomenon of DNA recognition which we show involves the sequential formation of at least three different types of enzyme–DNA complex. To understand this process, we must briefly describe the relationship between the sites on the DNA for host specificity, methylation and cleavage. The recognition sites are those sites on the DNA that confer susceptibility to the endonucleolytic action of the enzyme. They are relatively few in number for a given DNA and may be lost by mutation to yield a DNA molecule that cannot be cleaved, in vitro or in vivo, by the enzyme[8]. The methylation site seems to be a specific sequence of bases at the recognition site, since phage mutants that have lost their recognition sites also lose (again, both in vivo and in vitro) the ability to be methylated[9,10]. Furthermore, Horiuchi et al. have isolated small, specific DNA fragments from modified f1 DNA and found that of these fragments, only the two that contain the genetically mapped sB sites were methylated[11]. The situation concerning the cleavage sites is less clear. The enzyme does not cleave the DNA at the recognition sites and the number of possible cleavage sites is larger than the number of recognition sites[12]. Mapping studies with PM2 DNA have shown that the K cleavage sites are related to the readily denaturable regions of the genome (R.Y., T.A.B., W.E., and C.B., unpublished).

The interaction of EcoRK* with the host specificity site presents one of the most interesting aspects of the reaction mechanism because it is this interaction which will determine whether the enzyme will methylate or cleave the DNA. The site can exist in three possible forms: fully modified, fully unmodified or heteroduplex (one strand modified, the other not). We have previously shown that such heteroduplex DNA is not a substrate for restriction[1], and Vovis et al.[13] have shown that it is the best substrate for modification and that Ado–Met, ATP and

Mg^{2+} are required for optimal reaction. It is thus the recognition site that determines whether the enzyme will restrict or modify. If the site is unmodified, it will trigger the enzyme to its restricting mode, the enzyme will proceed to a cleavage site and restrict the DNA. If it is modified on one strand, the enzyme will be altered to its modifying form, and methylate the other strand. If it is fully modified, the enzyme will not recognise it, and will continue to scan other DNA molecules. The interactions of the enzyme with heteroduplex DNA are currently the object of extensive study in our laboratory. Both the recognition and cleavage complexes protect a small segment of DNA from DNase digestion. Sequencing of these fragments is currently in progress, and should clarify the location on the DNA of the recognition and cleavage complexes.

The restriction enzyme first breaks one strand followed some time later by a break on the opposite strand[1]. At this point, it no longer behaves as an enzyme, since having cleaved once, it does not do so again. Simultaneously with DNA cleavage, a vigorous ATP hydrolysis occurs, which continues for a long period of time[2]. Both observations can be readily reconciled if the restriction enzyme dissociated during cleavage to yield a species that would remain bound to the DNA. This complex would catalyse the ATP hydrolysis. Three observations lend support to this view: detection of a stable protein–DNA complex that hydrolyses ATP[14]; the lack of turnover (measured by both DNA binding and cleavage); and the complementation of a limit digest by either of two mutant restriction enzymes[5] to give a new round of endonuclease activity.

In conclusion, it is clear that the mechanism of *EcoRK* is highly complex. What is not readily apparent is the reason for such an elaborate machinery to carry out reactions that are carried out in a much simpler manner by other restriction and modification systems. It cannot be ruled out that the complexity may be related to its involvement in other reactions such as DNA replication or recombination.

[1] Meselson, M., and Yuan, R , *Nature*, **217**, 1110–1114 (1968).
[2] Yuan, R , Heywood, J , and Meselson, M., *Nature new Biol.*, **240**, 42–43 (1972).
[3] Haberman, A , Heywood, J , and Meselson, M., *Proc. natn. Acad. Sci U S.A.*, **69**, 3138–3141 (1972).
[4] Yuan, R., and Meselson, M , *Proc natn. Acad. Sci U.S A.*, **65**, 357–362 (1970)
[5] Hadi, S. M., and Yuan, R , *J. biol. Chem* , **249**, 4580–4586 (1974)
[6] Hadi, S M., Bickle, T A., and Yuan, R., *J biol Chem* , **250**, 4159–4164 (1975)
[7] Murray, N. E , Manduca de Ritis, P., and Foster, L. A., *Molec gen Genet* , **120**, 261–281 (1973)
[8] Murray, N E , Batten, P. L , and Murray, K , *J. molec Biol.*, **81**, 395–407 (1973).
[9] Smith, J D , Arber, W , and Kuhnlein, U , *J molec. Biol* , **63**, 1–8 (1972)
[10] Kuhnlein, U , and Arber, W., *J molec. Biol.*, **63**, 9–19 (1972).
[11] Horiuchi, K., Vovis, G. F , Enea, V , and Zinder, N. D., *J molec. Biol*, **95**, 147–165 (1975)
[12] Horiuchi, K., and Zinder, N. D , *Proc natn. Acad. Sci. U.S A* , **69**, 3220–3224 (1972)
[13] Vovis, G. F., Horiuchi, K., and Zinder, N D., *Proc. natn Acad. Sci U.S A* , **71**, 3810–3813 (1975)
[14] Linn, S , Lautenberger, J A., Eskin, B., and Lackey, D , *Fedn Proc* , **33**, 1128–1134 (1974).

letters to nature

Chemistry of the solar neutrino problem

ELECTRON neutrinos with energies of about 1–10 MeV are generally presumed to be produced by thermonuclear reactions in the Sun's core. Since 1967 an experiment has been under way on Earth to detect a subset of these neutrinos: argon atoms from the reaction

$$\nu_e + {}^{37}Cl \rightarrow {}^{37}Ar^+ + e^- \qquad (1)$$
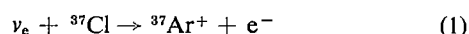
are chemically isolated, and the Auger electrons characterising their electron-capture decay are counted.[1] A sizeable discrepancy—the solar neutrino problem[2,3]—has appeared. The most recent observational results[4] imply an upper limit (1σ) of 1 SNU ($= 10^{-36}$ solar neutrino captures per target ${}^{37}Cl$ nucleus s^{-1}), whereas the latest theoretical expectation based on standard models[5] is 5.6 SNU.

Something is definitely awry in our understanding of solar structure theory, nuclear physics, neutrino physics, or the detection chemistry. A solution to the solar neutrino problem has been extensively sought in the first three of these areas[6,7] (see also refs 2 and 3), but these efforts have recently encountered severe constraints[8-10]. The last area—the chemistry of the detection experiment—is one of the more neglected aspects of the problem; here I shall attempt to rectify this imbalance.

In the solar neutrino experiment a tank of liquid tetrachloroethylene (C_2Cl_4) provides the target ${}^{37}Cl$ nuclei of equation (1). The number of ${}^{37}Ar$ nuclei in the tank, $N(t)$, is governed by the equation

$$dN/dt = R + b - (N/\tau) \qquad (2)$$

where R and b are the respective production rates resulting from solar neutrinos and background processes, and where τ ($=50.6$ d) is the electron-capture-decay lifetime of ${}^{37}Ar$. For $t \gg \tau$, the solution to equation (2) asymptotically approaches the limiting value, $N(\infty) = (R + b)\tau$. The detector parameters[1] yield $R\tau = 9.6$ ${}^{37}Ar$ nuclei per SNU; the cosmic-ray-induced background for the water-shielded tank is estimated[11] to be $b = 0.09$ ${}^{37}Ar$ nuclei d^{-1}. Therefore

$$N(\infty) = 9.6(SNU) + 4.5 \qquad (3)$$

where (SNU) denotes the actual capture rate of solar neutrinos in the tank of C_2Cl_4.

Standard theory[5] implies that (SNU) $\simeq 5.6$, so the anticipated steady-state population from equation (3) is $N(\infty) \simeq 58$ ${}^{37}Ar$ nuclei. The experimenters are attempting to extract fewer than 100 ${}^{37}Ar$ atoms from 390,000 l of tetrachloroethylene, to count them, and to determine the number produced by solar neutrinos in comparison with that arising from background processes. The complexity and marginal nature of the experiment lead me to put forward the hypothesis that the solar neutrino problem is solely an artefact of the chemistry of the detection technique. A direct corollary is that ${}^{37}Ar$ nuclei are, in fact, being produced by solar neutrinos in the tank at the rate of 5.6 SNU, so that $N(\infty) = 58$ ${}^{37}Ar$ nuclei. The experimental upper limit of 1 SNU then implies that only a small fraction of the ${}^{37}Ar$ in the tank is revealed in the output pulses of the Auger counter. (I shall use the loose terminology, 'chemical trapping', to indicate the fate of the undetected ${}^{37}Ar$ nuclei.)

Five experiments constrain this chemical hypothesis. First[1], a known volume of pure ${}^{36}Ar$ gas was dissolved in the tank

tetrachloroethylene, extracted by helium purging, and measured volumetrically; a 22-h extraction recovered 95% of the ^{36}Ar. Second (R. Davis, Jr, and J. C. Evans, Jr, unpublished), 612 ± 20 atoms of ^{37}Ar were mixed into the tank, the helium purge operated for three days, and the extracted ^{37}Ar detected in the Auger counter; it was inferred that the tank had contained 650 ± 50 (1σ) ^{37}Ar atoms.

These two experiments alone imply that neutral atoms of argon are not 'trapped' in Davis' system, even at the level of about 10^2 atoms. I note, however, that the product of reaction (1) is the cation ^{37}Ar$^+$ and not neutral ^{37}Ar. The remaining three experiments probe the possible 'chemical trapping' of this charged ^{37}Ar$^+$.

In the third experiment (see ref. 1), *in situ* irradiation of the tank liquid with fast neutrons produced ^{37}Ar$^+$ by the reaction

$$[n^0(\text{Ra–Be}) + {}^{35}\text{Cl} \to {}^{35}\text{S} + \text{p}^+ \text{ (fast)}$$
$$\downarrow$$
$$\to + {}^{37}\text{Cl} \to {}^{37}\text{Ar}^+ + n^0 \quad (4)$$
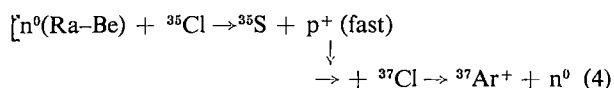
Positive identification of ^{37}Ar at the Auger counter showed the helium-purge recovery of ^{37}Ar atoms to be proportional to that of the carrier ^{36}Ar, and the production rate was inferred to be 7.5×10^{-7} ^{37}Ar nuclei per incoming neutron. This determination was not, however, absolute; most of the ^{37}Ar nuclei could well have remained 'chemically trapped' in the tank.

The fourth 'experiment' (see ref. 11) was the calibration of cosmic-ray-induced ^{37}Ar in Davis' tank. The standard chemical procedure indicated that the equilibrium ^{37}Ar activity of small tanks of C_2Cl_4 at six different depths in the Homestake gold mine scaled with depth as expected theoretically; extrapolation to Davis' main tank implied a background rate of $b = 0.09 \pm 0.03$ ^{37}Ar nuclei d^{-1}. These results are, however, also not absolute, since the theoretical curve was normalised to the data at a depth of 25 hg cm^{-2} (standard rock). Should 'chemical trapping' prove important, then the canonical background rate will become highly suspect.

Ion–molecule reactions leading to the bound species ArC$_n$Cl$_m$$^+$ were tested in the fifth experiment[12], in which a gas-phase mixture of ^{40}Ar and G_2Cl_4 (at 1 mmHg pressure) was ionised with 40–eV electrons and the products were detected with a quadrupole mass analyser. No ArC$_n$Cl$_m$$^+$ was found, and the results were completely dominated by charge transfer (neutralisation of ^{40}Ar$^+$ by electron pickup). To extrapolate these gas-phase findings to a liquid C_2Cl_4 environment ($\simeq 10^6$ times denser), however, is totally unwarranted.

From this investigation of experimental constraints I conclude that the chemical hypothesis remains a viable solution to the solar neutrino problem only if the ^{37}Ar$^+$ is 'chemically trapped' in Davis' system before neutralising to free ^{37}Ar atoms. This final loophole may be closed by another experiment now being performed by Davis (unpublished): a known number of $C_2Cl_3{}^{36}Cl$ molecules is put in his tank, the β-decay of ^{36}Cl produces a determinate number of ^{36}Ar$^+$, and the amount of ^{36}Ar recovered is measured

The evidence supporting my chemical hypothesis is purely circumstantial, but deserves mention. The clear signature of 58 ± 20 ^{37}Ar atoms in the tank at the end of run 27 was provided by the Auger counter[4,13]; equation (3) then implies that (SNU) = 5 6 ± 2.0. Contamination, cosmic-ray background variations, and statistical fluctuations are considered extremely unlikely causes[4,14], so the result of run 27 is generally attributed to a large ν_e burst from a galactic 'collapsing star' (see refs 4 and 14; also R. Davis, Jr, and J. C. Evans, Jr, unpublished). On January 4, 1974 (during run 32), however, a strong $\bar{\nu}_e$ burst was apparently detected in an independent experiment in the Homestake gold mine[15]. If this burst signalled a 'collapsar', then a large accompanying $\bar{\nu}_e$ flux can be expected[16], so it becomes difficult to understand the data of run 32 which implied only 9 ± 9 ^{37}Ar atoms in Davis' tank[4]. These observations can, however, be accommodated by my chemical hypothesis.

By what mechanisms may ^{37}Ar$^+$ become 'chemically trapped' in Davis' system? Tetrachloroethylene is an unsaturated molecule, and short-chain polymerisation of liquid C_2Cl_4 has been achieved[17] using MeV-γ-radiation; the ^{37}Ar$^+$ may induce polymerisation and trap itself within a solid polymer analogous to Teflon. The rare-gas atoms, krypton and xenon, occur in stable molecules[18] (for example, XeF$_6$), apparently partially because of electron-d-orbital interactions; since Ar$^+$ is isoelectronic to neutral chlorine there are 3d orbitals available which may permit 'back-binding', as in ClO$_4^-$ and in the 'π-d-complex intermediates'[19]. The existence of stable molecules containing argon has been suggested[20]. Finally, the stabilisation of ion–molecules involving Ar$^+$ and C_2Cl_4 (in bound excited states?) should be promoted by the strong inductive effects of Ar$^+$ and by cooperative relaxation processes in the liquid tetrachloroethylene environment. Chemical experiments to investigate some of these possibilities are currently underway.

KENNETH C. JACOBS*

Department of Astronomy,
University of Virginia,
Charlottesville, Virginia 22903

1 Davis, R., Jr, Harmer, D S , and Hoffman, K. C., *Phys Rev Lett.*, 20, 1205–1209 (1968)
2 Bahcall, J N., and Sears, R. L , *A Rev Astron Astrophys*, 10, 25–44 (1972).
3 Trimble, V., and Reines, F , *Rev mod Phys*, 45, 1–5 (1973)
4 Evans, J. C , Davis, R , Jr, and Bahcall, J N , *Nature*, 251, 486–488 (1974).
5 Bahcall, J N , *et al.*, *Astrophys J* , 184, 1–4 (1973).
6 Ulrich, R. K , *Astrophys J* , 188, 369–378 (1974)
7 Hoyle, F., *Astrophys J Lett.*, 197, L127–L131 (1975)
8 Reines, F , Sobel, H W., and Gurr, H. S , *Phys. Rev Lett* , 32, 180–181 (1974)
9 Rood, R T , and Ulrich, R K , *Nature*, 252, 366–368 (1974)
10 Hill, H A , *et al* , *Phys Rev. Lett* , 33, 1497–1500 (1974)
11 Wolfendale, A W , Young, E C. M., and Davis, R , Jr, *Nature phys Sci* , 238, 130–131 (1972)
12 Leventhal, J J , and Friedman, L , *Phys. Rev* , D, 6, 3338–3339 (1972).
13 Davis, R., Jr, and Evans, J C , *Proc 13th int Cosmic Ray Conf.*, 3, 2001 (Denver, 1973)
14 Pallister, W S , and Wolfendale, A. W , *Nature*, 251, 488–489 (1974).
15 Lande, K., *et al* , *Nature*, 251, 485–486 (1974).
16 Schramm, D N., and Arnett, W. D , *Phys Rev. Lett.*, 34, 113–116 (1975).
17 Miller, W , *et al.*, *J chem Soc* , 4485 (1961).
18 Holloway, J H., *Noble-Gas Chemistry* (Methuen, London, 1968)
19 Hendrickson, J. B , Cram, D J , and Hammond, G S , *Organic Chemistry*, third edition, 789–814 (McGraw-Hill, New York, 1970).
20 Pimentel, G C , *J chem Phys.*, 19, 446–448 (1951)

Ultraviolet laser sounding of the troposphere and lower stratosphere

WE report here measurements made with a ground-based, ultraviolet laser operating in the wavelength range 297–308 nm, which relate to the question of the atmospheric transmission between ground level and about 20 km, and particularly to the contributions of absorption by minor constituents There has been considerable concern in recent years about the biological effects of increased solar ultraviolet radiation at ground level associated with a reduction in the concentration of atmospheric ozone[1]. It seems that wavelengths between about 300 and 310 nm are most likely to produce erythema (sun burn) and skin cancer[2]. The effects of aerosols, clouds, non-absorbing haze or fog and other absorbing atmospheric gases need to be considered in addition to the effects of ozone and of Rayleigh scattering when examining the penetration of these radiations to the Earth's surface[3,4]. Except for the Junge layer[5] of aerosols which extends from about 12 to 22 km, and cirrus clouds,

these scattering and absorbing agencies are probably located in the lower troposphere, below about 5 km.

Our observations were made at Winkfield (60 m above sea level and 40 km west of central London) with a laser radar system (Table 1). The transmitter consisted of a flash-lamp-pumped dye laser, tuned with Fabry–Perot etalons, frequency-doubled by an ammonium dihydrogen phosphate (ADP) crystal orientated for critical phase-matching. The receiver used a 1–m aluminised mirror, various interference filters and a bialkali-cathode photomultiplier The absence

Table 1 Parameters of the ultraviolet laser radar system

Transmitter	
Wavelength range	297–308 nm
Linewidth	0.2 nm
Energy per pulse	approximately 0.2 mJ
Beam divergence	0.2 mrad × 2 mrad
Pulse duration	5 µs
Pulse repetition rate	1 s^{-1}
Receiver	
Mirror area	0.6 m^2
Beamwidth	2 mrad
Separation from transmitter	4 m

of spurious signals from any photomultiplier overloading or signal-induced noise[6] was confirmed from soundings of cloud observed in the 5–10 km height range, above which no signal was detected. Rotation of the ADP crystal from the index-matching angle showed that the blocking of the receiver filters was sufficient to reject radiations scattered from the fundamental beam.

Figure 1 shows the results obtained on five nights when the laser was tuned to 308 nm. Measurements were restricted to periods when the sky was apparently clear, and the number of laser firings each night varied between about 100 and 1,000. The signal observed in each height channel was compared with that expected for Rayleigh scattering from the standard atmosphere for midlatitude winter conditions[7], the resulting relative signal being normalised at 5.5 km. Attenuation of the beam because of Rayleigh scattering was allowed for in the calculation of the expected signal. The results obtained on different nights show height variations which are either consistent with Rayleigh scattering (February 24, 1975; Fig. 1e), show decreases with increasing height by up to a factor of 4 (January 12, January 31 and February 1, 1975; Fig. 1b, c, and d, respectively), or show small enhancements (December 30, 1974; Fig. 1a). Changes in the total molecular density cannot account for the night-to-night variability, so the results indicate either appreciable scattering and absorption by aerosols in the heightrange 5–20 km, or absorption of the radiation by a minor molecular constituent. This would probably be ozone, as the concentrations of sulphur dioxide, hydroxyl and other absorbing constituents above 5 km are expected to be too low to give detectable attenuation.

In an attempt to measure the height distribution of ozone, observations were also carried out using two wavelengths· 308.0 nm and 303.5 nm. These correspond to a ratio of 1 : 1.7 in the ozone absorption cross section[8,9] and are close to minima in the sulphur dioxide cross section[10]. As the scattering and absorption characteristics of aerosols are not expected to vary significantly over this wavelength range, the difference in attenuation would arise from absorption by ozone. The results obtained are shown in Fig. 2 where the points for each wavelength have been separated horizontally for ease of presentation. For heights between 5 and 15 km, the two sets of results are each consistent with a straight line variation on the logarithmic scale. The ratio of slopes of these lines is closer to unity than would be

expected for absorption by ozone alone, but the difference in slope is found to correspond to ozone concentration of about 4×10^{17} m^{-3}, a height-independent which is within the range of measurements by other methods[11].

Signals received from heights below about 5 km could not be interpreted, mainly because of uncertainty in the overlap of the transmitting and receiving beams. An estimate of the transmission coefficient below 5 km after allowing for Rayleigh scattering can, however, be obtained by comparing the observed signal from 5–6 km with that calculated from a knowledge of the ultraviolet pulse energy and overall receiver efficiency. A comparison of the results obtained for different wavelengths is relatively free from the uncertainties associated with the transmission coefficient obtained for each separately. From measurements carried out with a greater range of wavelengths on February 24, it was found that the two-way transmission coefficient below 5 km at 297 nm was lower by a factor of 10 than that at 308 nm. This cannot be explained by the attenuation effects of aerosols alone and absorption by molecular oxygen is negligible as the cross section is less than 10^{-29} m^2 (ref. 12). If the ratio of transmission at 297 and 308 nm is attributed to absorption by sulphur dioxide in the 0–1 km height range, a concentration of 4×10^{19} m^{-3} is implied (1 p.p.m.), which is very unlikely. If absorption by ozone in the height range 0–5 km were responsible, an average concentration of about 5×10^{18} m^{-3} would be required. This is an order of magnitude greater than that deduced for the 5–15 km height range, and is about five times larger than the mean background concentrations observed near the ground in north-western Europe during summer and is approached only during periods of photochemical air pollution[13,14]. The

Fig. 1 Observed signals at 308 nm on five nights between December 1974 and February 1975, relative to those expected for Rayleigh scattering: a, December 30; b, January 12; c, January 31; d, February 1, e, February 24. Error bars indicate the standard deviations corresponding to the photon count for each height channel
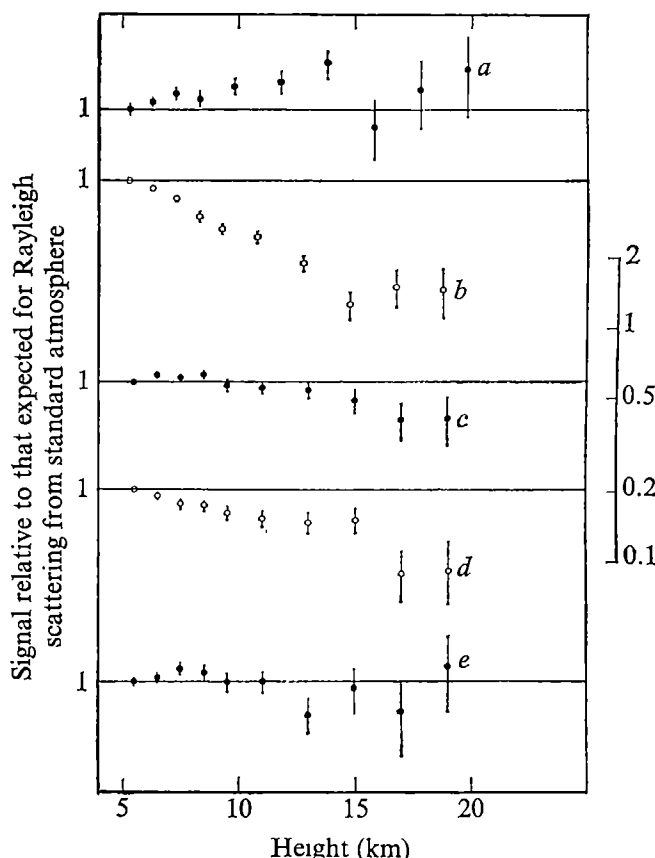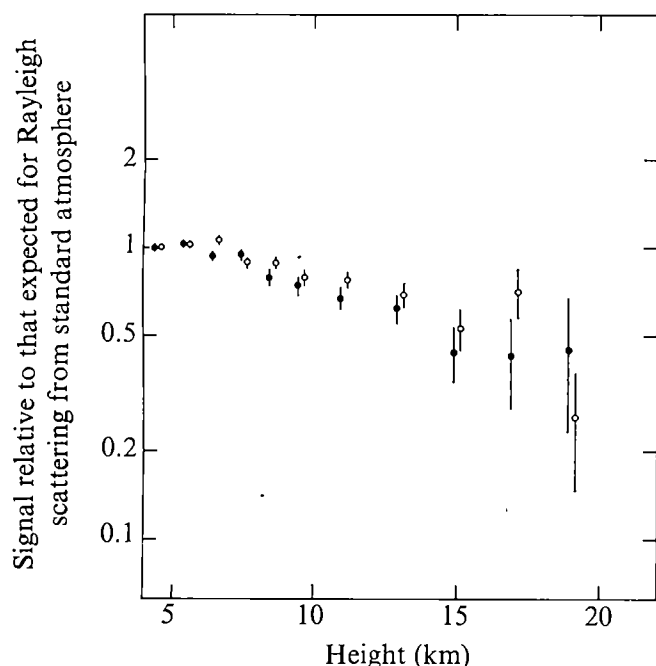
Fig. 2 Observed signals at 303 5 nm (●) and 308.0 nm (○) for February 27, 1975, relative to those expected for Rayleigh scattering Error bars indicate the standard deviations corresponding to the photon count for each height channel.

results can be better interpreted in terms of absorption by water vapour in the height range 0–5 km; a difference in absorption cross section of about 10^{-27} m^2 between 297 and 308 nm would be required for that to occur, but no accurate measurments of cross section are available for this range of wavelengths.

There is considerable scope for the further development of this technique of ultraviolet laser sounding of the atmosphere. With the production of new frequency-doubling materials suitable for 90° phase-matching[15], it should become possible to increase substantially the available ultraviolet pulse energies. The accuracy and height resolution of the present type of measurement should thereby be improved, and the height range extended. In addition, measurements of the concentrations of certain constituents by resonance scattering or fluorescence should become possible.

A. J. GIBSON

L. THOMAS

Science Research Council,
Appleton Laboratory,
Ditton Park,
Slough SL3 9JX, Berkshire, UK

[1] Hammond, A L, and Maugh, T H, *Science*, **186**, 335–338 (1974).

[2] Robertson, D F, in *Biological Effects of Ultraviolet Radiation* (edit by Urbuch F), 619–23 (Pergamon, Oxford, 1969)

[3] Halpern, P., Dave, J V, and Braslau, N, *Science*, **186**, 1204–1208 (1974)

[4] Nack, N L, and Green, A E S, *Appl Opt*, **13**, 2405–2415 (1974)

[5] Junge, C E, *An Chemistry and Radioactivity* (Academic, New York, 1969)

[6] Pettifer, R E W, and Healey, P G, *J Phys., E*, **7**, 617–620 (1974)

[7] Cole, A E, Court, A, and Kantor, A J, in *Handbook of Geophysics and Space Environments* (edit by Valley, S L), ch.2 (McGraw-Hill, New York, 1965)

[8] Inn, E C Y, and Tanaka, Y, *J. opt Soc Am*, **43**, 870–873 (1953)

[9] Vigroux, E, *Annln Phys*, **8**, 709–762 (1953)

[10] Thompson, A, Harteck, P, and Reeves, R R, *J geophys Res*, **68**, 6431–6436 (1963)

[11] Dutsch, H U, *Can J Chem.*, **52**, 1491–1504 (1974)

[12] Shimazaki, T, and Laird, A. R, *J geophys Res*, **75**, 3221–3235 (1970)

[13] Atkins, D H F, Cox, R A, and Eggleton, A. E. J, *Nature*, **235**, 372–376 (1972)

[14] Cox, R A, Eggleton, A E J, Derwent, R G, Lovelock, J E, and Pack, D H., *Nature*, **255**, 118–121 (1975)

[15] Adhav, R S, and Wallace, R W, *IEEE J quant. Electron*, **9**, 855–856 (1973).

Petroporphyrins as indicators of geothermal maturation

WE report here two new sets of data which provide evidence of the occurrence of the postulated conversion of deoxyphylloerythroetioporphyrin (DPEP) to etioporphyrin. Simulated geothermal maturation in the laboratory demonstrates that DPEP–etio conversion occurs proportionally to the severity of the treatment, and an analysis of petroporphyrins from a suite of genetically related petroleums and their source rocks reveal that the DPEP:etio ratio is inversely proportional to the presumed severity of the geothermal history of the sample.

Alkyl petroporphyrins occur in a wide range of geological materials[1,2] where they are usually present as two major homologous series of the deoxyphylloerythroetioporphyrin (DPEP) and etio type. Initially, the DPEP compounds containing the isocyclic ring were thought to be representative of, and derived from, chlorophylls, whereas the etio homologues, with no isocyclic ring, were considered to be remnants of the haem type pigments[3]. But though chlorophyll pigments exist in far greater abundance than haem pigments in the biosphere[4], DPEP and etio porphyrins[5] frequently occur in similar amounts in geological samples, and Corwin suggested[4] that the conversion of

Fig. 1 Relative abundance of metallopetroporphyrins (VO) from thermally altered (210 °C for 0 (*a*), 50 (*b*), 100 (*c*), 150 (*d*) and 385 (*e*) h) La Luna (Mara) extract obtained by mass spectrometric dermination of M$^+$ at the following masses: DPEP (solid line) = $(457+n14)$ a.m.u ; etio (dashed line) = $(459+n14)$ a.m.u,; where $n = 1$ to 13 (C_{27} to C_{39}). The mass spectra were collected by computer controlled gas chromatography–mass spectrometry; because of instrument background low abundance ions are occasionally not recorded (for example, C_{37}, C_{38}, and C_{39} in the 50-h (*b*) and 100-h (*c*) heating experiments)
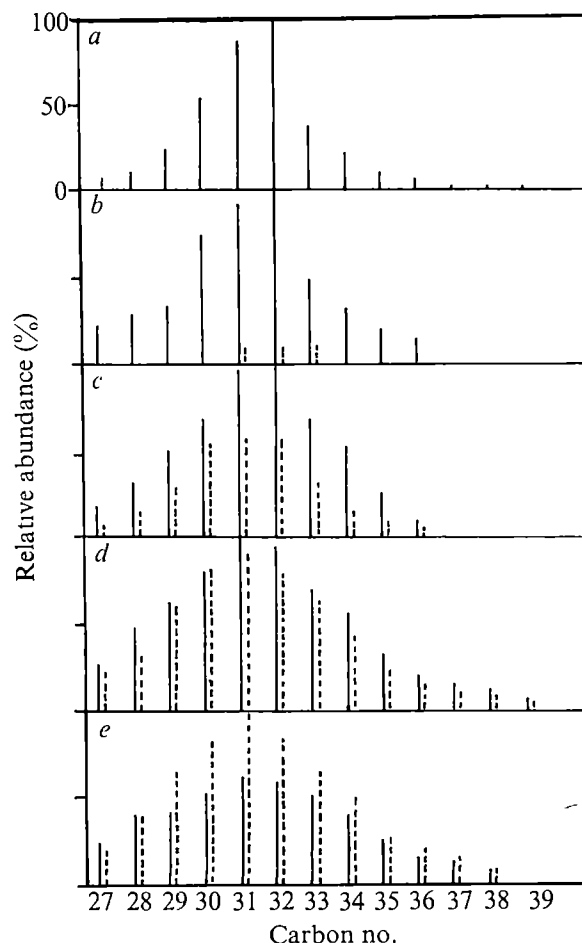
Table 1 Characteristics of petroporphyrins and source rocks from the Maracaibo Basin

| Sample | | Current geological setting | | | | Petroporphyrins | | |
| | | | | | | Carbon no. of most abundant molecular ion | | |
	Type	Age*	Depth (m)	Temperature (°C)	Content‡ (p.p.m.)	DPEP	etio	DPEP/etio
a La Luna (Mara)	Source rock	Cretaceous	outcrop	<30†	55,900	32	—	≫100 ·
b Boscan (BN-4)	Petroleum	Eocene reservoir	2,050–2,230	71	3,540	32	31	1.56
c La Paz (P187-Z)	Petroleum	Jurassic fracture	3,300–3,450	95	628	31	30	0.56
d La Luna (36E-2)	Source rock	Cretaceous	4,410–4,550	135	2,730	31	30	0.56

*The two oils are believed to have originated in the La Luna Formation (Cretaceous) but are currently found in the host formations indicated[13].
†No information concerning maximum depths of burial or temperature history is available but low reflectivity ($R_{av} = 0.19$) of vitrinite suggests a mild geothermal history. (We thank Dr M. Jones, Organic Geochemistry Unit, University of Newcastle, for this measurement.)
‡Metalloporphyrin content (p.p.m.) in oil or organic extract.

DPEP to etio homologues must occur in the geological environment. Indirect evidence of the conversion of DPEP to etio has been obtained from a comparison between petroporphyrins recovered from a retorted shale oil and those from the original shale[6]. The retorted oil contained a large proportion of etio type homologues, suggesting that conversion did occur during the retorting. The drastic temperatures required for the retorting process are, however, significantly higher than those (about 50–180 °C) normally experienced in deeply buried sediments, which effect the geothermal maturation of entrapped or dissolved petroporphyrins and other organic materials. Evidence of the conversion has also been provided by the thermal alteration, in the laboratory, of a crude petroleum containing metallopetroporphyrin to which etioporphyrin III had been added as an internal reference compound[7]. Other studies which have simulated geothermal maturation[8-11] have been restricted to porphyrins of the etio skeleton; they could not, therefore, provide information about the fate of the isocyclic ring of DPEP homologues or about the occurrence of the DPEP to etio conversion.

The geological materials used (Table 1) in this study are from, or are genetically related to, the Cretaceous La Luna formation of the Maracaibo Basin in western Venezuela. This formation is extensive and source beds in it are believed to have generated the major part of the oil produced in the Maracaibo area[12]. One of the most remarkable characteristics of these shaly limestones is the high porphyrin content (up to 55,000 p.p.m. of the organic extract); it has, in fact, been used to establish source rock–crude oil relationships for the oils from the Maracaibo Basin[13].

The simulated geothermal maturation was performed on the total organic extract of the least mature shale (Table 1*a*). The toluene–methanol (3:1) extract was deposited on prewashed (redistilled hexane B.D.H.) and vacuum-dried bentonite to give a concentration of about 2,790 p.p.m. of metallopetroporphyrin (98% vanadyl). Bentonite, a montmorillonitic clay mineral was chosen as the organic free matrix for thermal alteration experiments. Results obtained using this material rather than limestone should be more generally applicable to the geothermal fate of porphyrins in argillaceous sediments.

Aliquots of coated bentonite were placed in heavy-walled glass tubes (15 × 150 mm), flushed with oxygen-free nitrogen and sealed under vacuum. The sealed tubes were stored in the dark at 210 °C; individual tubes were removed at successively longer time intervals from 0 to 385 h. After cooling, the bentonite was extracted with toluene–methanol (3:1) and the metallopetroporphyrins were isolated, and purified using thin-layer chromatography (silica gel H, 0.5 mm, toluene–methylene chloride 1:1). Recoveries of total metallopetroporphyrins after thermal treatment varied from 81% (0 h) to 19% (385 h experiment). The thermal degradation of metallopetroporphyrins was found to be first order ($t_\frac{1}{2} = 166$ h). The extracts were examined using mass spectrometry: a computerised data handling system[14] monitored and collected the spectra in real time.

Bar diagrams (Fig. 1) show the relative abundance of the molecular ions of metallopetroporphyrins isolated after various times of thermal treatment. The zero-time extract exhibits a single homologous DPEP series of carbon number C_{27}–C_{39}, maximising at C_{32}. After 50 h an etio series could just be detected, after 100 h this was prominent and after 385 h the abundance of this series exceeded that of the now much-diminished DPEP compounds. Thus, the DPEP series seems to have undergone extensive thermal conversion to the etio series. The most abundant DPEP homologue remaining was then C_{31}, indicating that a second maturational process involves loss of alkyl groups. Dealkylation is a well known feature of geothermal maturation of other geolipids[15] and has been recorded following the laboratory thermal alteration of etioporphyrins[8-11].

In the second part of this study petroporphyrins from two crude oils and from the toluene–methanol (3:1) extracts of two

Fig. 2 Relative abundance of petroporphyrins isolated from geological samples described in Table 1. Mass spectrometric determination of M⁺ occurring at the following masses: DPEP (Solid line) = (392+*n*14) a.m.u.; etio = (394+*n*14) a.m.u.; where *n* = 1–13 (C_{27}–C_{39}). *a*, La Luna (Mara), *b*, Boscan BN4, *c*, La Paz P187-Z; *d*, La Luna 36E-2.
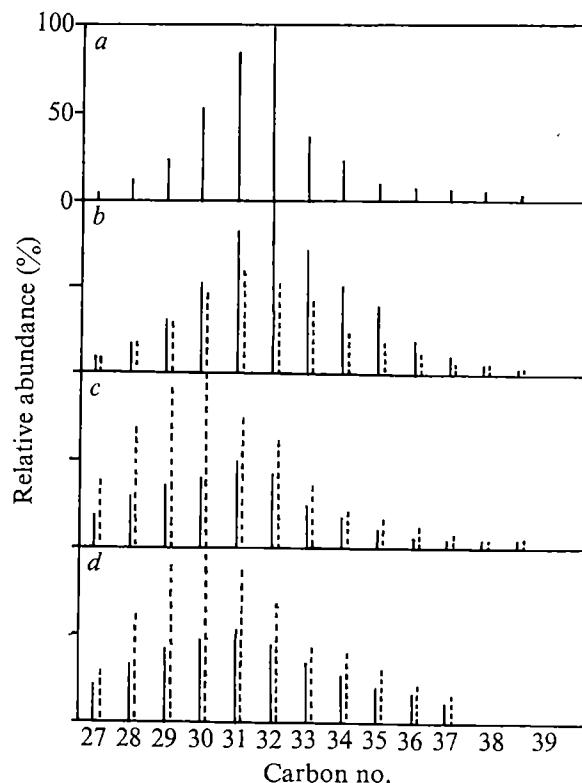
different samples of their presumed source rock (Table 1) were isolated using demetallation-extraction with methanesulphonic acid (4 h at 110 °C) and were analysed using mass spectrometry[16]. Figure 2 presents the relative abundances of the petroporphyrins of these samples; the samples increasing in maturity (based on geological information) from top to bottom of the diagram. As the geothermal maturity increases there is an increase in the relative abundance of the etioporphyrins and a shift to the lower carbon number of the most abundant petroporphyrin observed (Table 1). The decrease in the DPEP : etio ratio would suggest that during geothermal maturation DPEP species are converted into etio homologues. A general trend observed[17] within a group of non-genetically related geological materials suggests that the DPEP : etio ratio decreases with the depth of burial.

The DPEP–etio conversion suggested for the Maracaibo Basin samples parallels the conversion observed in the thermal alteration experiment. In each case the maxima of the two homologous series occur either at the same carbon number or with the abundance of etio homologues maximising one carbon atom lower than the DPEP homologues. Similar distributions have been observed in a wide range of naturally occurring petroporphyrins[1]. The thermally induced conversion of DPEP to etio porphyrins may be widespread in the geological environment and could account for the abundance of etio homologues in crude oils and sedimentary rocks. The decrease in the DPEP: etio ratio with increasing maturation could be a useful measure of the extent of maturation of genetically related geological materials.

We thank the Empresa Nacional del Petroleo (Chile), the Ford Foundation, the University of Riyadh and the Nuffield Foundation for financial support. The geological samples were provided by the late Dr J. A. Gransch (Shell, Holland) and by Mr E. E. Hagan (Chevron Oil Company of Venezuela).

Borys M. Didyk
Yousif I. A. Alturki
Colin T. Pillinger
Geoffrey Eglinton

Organic Geochemistry Unit,
School of Chemistry,
University of Bristol,
Bristol BS9 1TS, UK

1 Baker, E W , Yen, T F., Dickie, J P , Rhodes, R E , and Clark, L. F., *J. Am chem Soc.*, **89**, 3631-3638 (1967).
2 Hodgson, G. W , Hitchon, B., Taguchi, K , Baker, B. L , and Peake, E., *Geochim. cosmochim Acta.*, **32**, 737-772 (1968)
3 Treibs, A , *Angew. Chem* , **49**, 682-686 (1936)
4 Corwin, A H , *Proc fifth World Petrol Congr* , **5**, 119-129 (1959)
5 Baker, E. W , in *Organic Geochemistry—Methods and Results* (edit by Eglinton, G , and Murphy, M J. T), 464-497 (Springer, Berlin and Heidelberg, 1969).
6 Morandi, J R , and Jenson, H B , *J. chem engng Data*, **11**, 81-88 (1966).
7 Alturki, Y I A , thesis, Univ Bristol (1972).
8 Casagrande, D J , and Hodgson G W , *Nature*, **223**, 123-124 (1971).
9 Casagrande, D J , and Hodgson, G W., *Geochim. cosmochim Acta*, **38**, 1745-1758 (1974).
10 Bonnett, R , Brewer, P , Noro, K , and Noro, T., *Chem Commun* , 562-563 (1972).
11 Yen, T F , Boucher, L. J., Dickie, J. D , Lyman, E. C , and Vaughan, G. B , *J Inst. Petrol* , **55**, 87-99 (1969).
12 Hedberg, H D , *Bull Am Ass Petrol Geol.*, **48**, 1755-1803 (1964)
13 Gransch, J A , and Eisma, E , in *Advances in Organic Geochemistry, 1966*, (edit by Hobson, G D , and Speers, G. C), 69-86 (Pergamon, Oxford, 1970)
14 Eglinton, G , Zoro, J A , and Simoneit, B R , *Proc. R. Soc* **B189**, 415-442 (1975)
15 Maxwell, J. R., Pillinger, C T , and Eglinton, G., *Q Rev. Chem Soc* , **25**, 571-628 (1971)
16 Alturki, Y. I A., Eglinton, G , and Pillinger, C T , *Advances in Organic Geochemistry*, 1971 (edit by von Gaertner, H R , and Wehner, H), 135-150 (Pergamon, Oxford, 1972).
17 Yen, T F., and Silverman, S R , *Am chem Soc Prepr Div. Petrol Chem.*, **14**, E-32 (1969).

Effect of climate on the magnetic susceptibility of soils

The magnetic susceptibility of soils derived from sedimentary rocks is normally significantly higher than that of the parent rock. Le Borgne[1,2] has suggested that this enhanced susceptibility of the soil is due to the *in situ* conversion of the iron oxides from an antiferromagnetic form such as haematite (α Fe_2O_3)

or goethite (α FeOOH) to the ferrimagnetic form, maghaemite (γ Fe_2O_3). He also proposed two possible mechanisms involving a reduction process followed by reoxidation to maghaemite. In the first (fermentation mechanism), the reduction occurs as a result of the decay of organic matter in the soil in anaerobic conditions achieved during wet periods, and reoxidation to maghaemite occurs in the aerobic conditions prevailing during subsequent dry periods. In the second (heating mechanism) the burning of organic material produces the temperature increase and reducing atmosphere necessary for the reduction to magnetite (Fe_3O_4) in a thin layer of soil underlying the fire and reoxidation occurs during the cooling down of the fires when air enters the system.

On the basis of the heating mechanism, the percentage of the iron oxide in the soil which has been converted *in situ* to maghaemite (that is, the percentage conversion) is given by the ratio of the observed magnetic susceptibility (χ_O) to the susceptibility (χ_N) after heating the soil in a laboratory furnace at 550 °C in an atmosphere of nitrogen followed by air[3]. Although pure maghaemite converts to haematite at temperatures above 350 °C, maghaemite doped with, for example, sodium, aluminium or magnesium is stable up to very much higher temperatures[4]. Therefore, since the iron oxides in soils are in a finely divided form and in intimate contact with the clay minerals, it is assumed that the laboratory heating converts the iron oxides to a thermally stable, impure maghaemite. Support for this hypothesis has been provided by Le Borgne[1] who has shown that soils with a high magnetic susceptibility exhibit an exothermic DTA peak, characteristic of maghaemite, at about 700 °C. Furthermore, X-ray diffraction measurements on the laboratory heated soils have established that either maghaemite or magnetite is present, and in view of the final oxidising stage in the heating, the latter alternative is unlikely.

Magnetic susceptibility measurements (Table 1) were undertaken, using an a.c. bridge system[5] operating at 1 kHz, on soils (0–20 cm; A horizon) derived from a wide range of sedimentary rock deposits (Triassic–Miocene) in England and Italy, as well as on a small selection of soils from other parts of the Mediterranean zone and from various tropical regions.

Table 1 Magnetic susceptibility ranges for soils

Region	Geology	Magnetic susceptibility ($\times 10^{-8}$ kg^{-1})	
		Observed (χ_O)	After heating (χ_N)
England	Limestone	15–90	1,000–5,000
England	Sandstone, clay	10–180	600–8,000
Italy	Marly limestone, marl	10–80	600–3,000
Italy	Sandstone, clay	5–30	600–2,000
Italy	Limestone	30–500	200–4,000
Mediterranean	Limestone	150–600	1,500–3,000
Mediterranean	Calcareous alluvium	30–400	500–6,000
Tropics (wet and dry)		15–500	200–8,000
Tropics (rainy)		5–15	700–1,000

The percentage conversions (that is, $100\chi_O/\chi_N$) observed for the English soils were normally less than 5%, irrespective of the type of sedimentary rock from which they were derived (Fig. 1*a*). In contrast, the percentage conversions observed for the Italian soils differed widely depending on the associated rock-type. Soils developed on marly limestones, marls, sandstones and clays, again normally exhibited percentage conversions of less than 5% (Fig. 1*b*) whereas those developed on hard, as opposed to marly, limestones exhibited significantly higher percentage conversions extending up to 50% (Fig. 1*c*). These differences in percentage conversion cannot be satisfactorily explained in terms of the heating mechanism as it is extremely unlikely that the limestone soils from Italy have been consistently subjected to more extensive burning than either the other

Italian soils or the English soils. Instead, it seems probable that the higher percentage conversions associated with the limestone soils from Italy arise because the fermentation mechanism is more extensively activated as a result of the combined effect of the prevailing Mediterranean climate and the high permeability of the limestone substratum. Anaerobic (reducing) conditions are achieved during the humid winters and the necessary aerobic (oxidising) conditions are achieved during the subsequent hot dry summers in the case of the well-drained soils which are developed on limestones.

Further evidence for the role of climate is provided by the fact that the limestone soils from Apulia, the Orbetello region and the southern Apennines, which are mainly of the red Mediterranean type[6] (that is, terra rossa) exhibit higher percentage conversions than those from the central Apennines which are mainly rendzinas (Fig. 1c). Again, it seems probable that this difference is because more sustained aerobic conditions are achieved during the summer months in the former areas as a result of lower rainfall and higher temperatures.

The percentage conversion data for soils developed on limestones and for calcareous alluvia from Greece, Crete and Turkey (Fig. 1d) confirm that high values for this parameter are not peculiar to Italy but seem to be generally characteristic of well-drained soils in the Mediterranean climatic zone. Similarly, the data for tropical soils from Nigeria, Gambia, Zambia, British Honduras and North Borneo provides further evidence for the role of climate in determining the percentage conversions achieved in soils (Fig. 1e). The majority of the soils from those regions in which a pronounced dry period occurs (that is, the

Fig. 1 Histograms showing the distribution of values for the percentage conversion for: a, English soils derived from limestones (shaded), and sandstones and clays (blank); b, Italian soils derived from marly limestones and marls (shaded), and sandstones and clays (blank); c, Italian soils derived from limestones in Apulia, the Orbetello region and the southern Apennines (shaded), and the central Apennines (blank); d, Mediterranean soils derived from limestones (shaded) and calcareous alluvia (blank); e, soils from wet and dry tropics (shaded) and rainy tropics (blank).

wet and dry tropics) again exhibit percentage conversions in excess of 5%, the characteristic red tropical soils being predominant in this group. In contrast, the soils from those regions in which no definite dry period occurs (that is, the rainy tropics) exhibited percentage conversions of less than 2%.

These results suggest that there is a high percentage conversion of iron oxide to the ferrimagnetic form, and a concomitant high magnetic susceptibility (Table 1) in well-drained soils developed in regions with a climate that includes a pronounced, hot, dry period. It is, therefore, possible that magnetic susceptibility measurements could be used to identify those palaeosols in, for example, England which were formed in warmer dryer climatic conditions than exist at the present time. In interpreting the magnetic susceptibility of soils it must, however, always be remembered that high percentage conversions can also be produced by burning associated either with the clearance of land for agriculture or with human habitation. For example, in England, although normal soils exhibit low percentage conversions (Fig. 1a), soils from archaeological sites (that is, topsoils, pit and ditch fillings) exhibit significantly higher percentage conversions in the range 4–20% (ref. 7).

We thank Dr C. Mullins, Mr J. A. Varley and Dr C. Vita-Finzi for providing soil samples. This work was supported in part by a grant from the Italian Ministry of Foreign Affairs.

M. S. TITE

Department of Physics,
University of Essex,
Wivenhoe Park,
Colchester CO4 3SQ, UK

R. E. LININGTON

Fondazione Lerici Prospezioni Archeologiche,
Rome, Italy

1 Le Borgne, E., *Annls Geophys.*, 11, 399–419 (1955).
2 Le Borgne. E., *Annls Geophys*, 16, 159–195 (1960)
3 Tite, M. S , and Mullins, C , *Archaeometry*, 13, 209–219 (1971)
4 Stacey, F D., and Bannerjee, S. K., *The Physical Principles of Rock Magnetism*, 92–95 (Elsevier, Amsterdam, 1974)
5 Scollar, I., *J. scient. Instrum* , 1, 781–782 (1968).
6 *Soil Map of Europe* (1 .2,500,000), (Food and Agricultural Organisation of the United Nations, Rome, 1966)
7 Tite, M S., *Archaeometry*, 14, 229–236 (1972)

New interpretation of dielectric loss peaks

ALTHOUGH the dielectric loss peaks in many materials are known to show a frequency dependence which departs radically from the ideal Debye shape, the interpretation of their temperature dependence is carried out implicitly within the framework of the Debye mechanism. This cannot produce a good physical insight into the situation and here I look at it from a new standpoint.

The frequncy dependence of loss, χ'' (ω), in many dipolar materials may be expressed by the empirical formula[1,2]:

$$1/\chi''(\omega) = (\omega/\omega_2)^{-m} + (\omega/\omega_1)^{1-n} \qquad (1)$$

where ω_1 and ω_2 are generally, thermally activated parameters, and the exponents m and $(1 - n)$ are both smaller than unity and decrease with decreasing temperature. This law of reciprocal addition gives a loss peak at a frequency ω_p, which increases with temperature. In many dielectric materials in which the polarisation is caused by hopping charges rather than by dipoles the peak is not readily observable because of the onset of direct current conduction, but the loss is still given by the second term in equation (1). I shall refer to this as the 'universal law of the dielectric behaviour'.

This law implies that the ratio of the imaginary to the real parts of the susceptibility is independent of frequency

$$\chi''(\omega)/\chi'(\omega) = \cot(n\pi/2) \qquad (2)$$
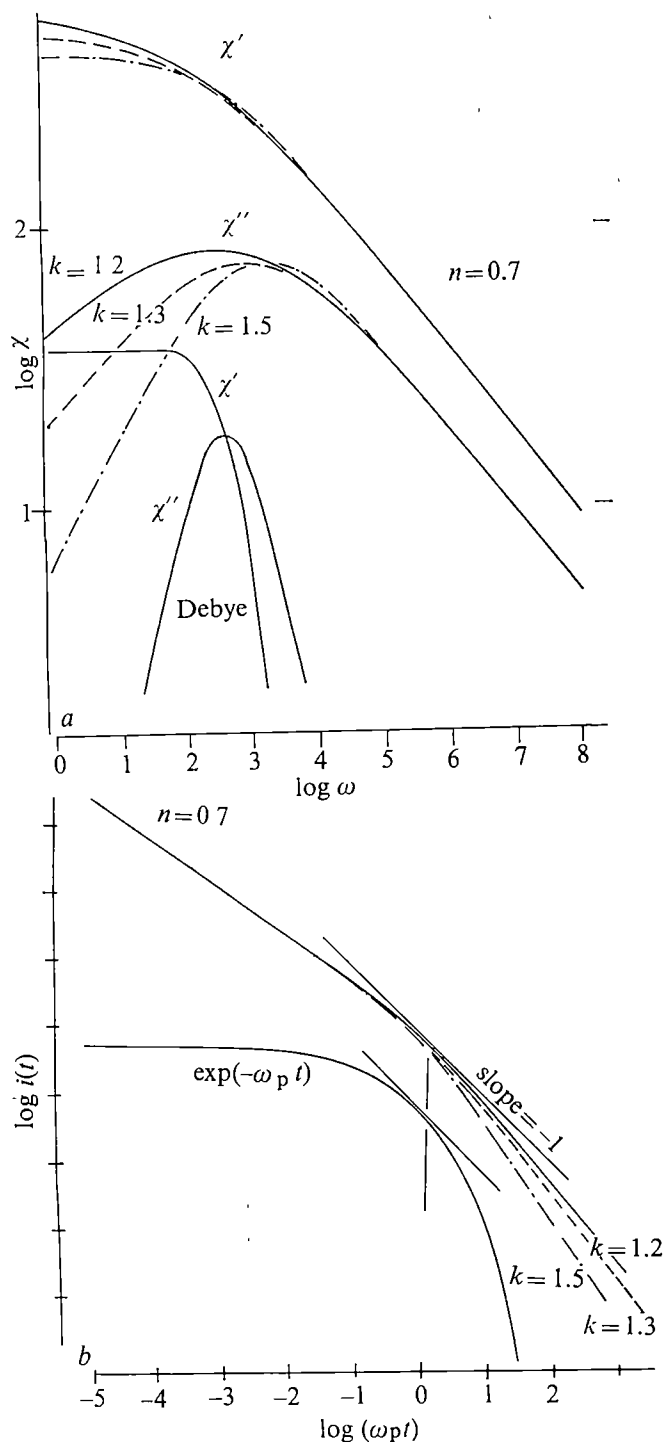
Fig. 1*a* Frequency-dependence of loss; *b*, time-dependence of current under step-function electric field excitation. Both correspond to equation (4), with $n = 0.7$ and values of $k = 1.2$, 1 3 and 1.5. The χ' (ω) and χ'' (ω) curves are Fourier-transformed numerically from equation (4) and show the parallel slope on the high-frequency side of the peak and the effect of the exponent on the positive slope and on the half-width of the peak. The pure Debye characteristics are shown on the same frequency scale in *a* and the exponential response on the same time scale in *b*. The vertical scales in *a* and *b* are scaled by arbitrary factors.

and leads to the physical interpretation that the ratio of energy lost per cycle to energy stored per cycle is constant, in marked contrast to the Debye mechanism for which it is equal to $\omega\tau$, where τ is the appropriate relaxation time. Any model satisfying this energy criterion must necessarily follow the universal law as a consequence of the Kramer–Kronig relationship. A physical mechanism which has the required properties and which should

be very widely applicable to both dipolar and hopping charge systems requires two assumptions: first, that charges or dipoles do not move smoothly but in discontinuous hops or jumps between preferred positions or orientations; second, that interactions with other charges of dipoles give rise to partial screening which can only follow the abrupt transitions with a finite delay.

The second term in equation (1) has, therefore, a simple interpretation in the frequency domain. The physical significance of the first term is less clear but I have suggested[3] that the gradual decrease of loss as $\omega \to 0$ arises because the perturbation of the system from its thermal equilibrium condition becomes progressively slower.

In the accepted interpretation of the temperature dependence of ω_p, the loss peak is treated as though it were a simple Debye peak[4]. The fact that its width at half-height may be two to eight times that of a Debye peak is left out of consideration, even though it may be acknowledged that the shape, and therefore the position of the peak, is determined by some distribution function, $g(\tau)$, of Debye relaxation times.

The concept of $g(\tau)$ is superficially very plausible—there is no doubt that physical systems must contain distributions of parameters rather than show strictly discrete values, especially in disordered materials and where stochastic processes govern the behaviour. In certain simple systems—for example, those involving multiple allowed orientations of molecular dipoles[5] or systems in which localised electrons execute multiple hops (V. Halpern, private communication)—it is possible to calculate exactly the distributions involved. Very complicated situations may arise for such cases as polar sidegroups on folded polymeric chains, but no rigorous analysis is possible in those cases[6].

What seems to have happened with the passage of time, however, is that the otherwise plausible, if restricted, concept of $g(\tau)$ became the universal explanation of all phenomena which did not fit into the idealised pattern, thus acquiring gradually the status of an unquestionable truth. The first point I challenge, therefore, is the scientific justification of the concept of the distribution of relaxation times in its application to the interpretation of dielectric data.

My argument is based on the universality of the observed behaviour[1,2] covering materials from plastics, through glasses, metal oxides, organic long-chain molecules and on to sand aggregates—seemingly regardless of their physical structure, chemical combination and bondage, or of the nature of the prevailing charged species responsible for polarisation (for example dipolar, electronic or ionic). It is completely implausible that the distribution of relaxation times in all of those diverse systems should happen accidentally to be such as to give the observed behaviour provided by the second term in equation (1). Having proposed a physical mechanism for the interpretation of this part of the spectrum[1], I turn here to the physical mechanism governing the region of the loss peak and below.

The precise form of the first term in equation (1) is of little significance here and the essence of my argument is not affected by it.

I propose that a more direct insight into the physical nature of the loss peaks may be obtained from an analysis of the time-domain response, $i(t)$, of a dielectric to step function electric field excitation. For an ideal Debye system:

$$i(t) \propto \exp(-t/\tau) \tag{3}$$

and the response corresponding to the empirical law is:

$$i(t) \propto [(t\omega_p)^n + (t\omega_p)^k]^{-1} \tag{4}$$

where the exponent, n, is the same as in equation (1) and $k > 1$.

Figure 1 shows a set of corresponding ω-domain and t-domain responses for an ideal Debye system and for the empirical behaviour, with $n = 0.7$ and with three values of k: 1.2, 1.3 and 1.5, respectively. In the latter case, ω_p corresponds to the time at which the slope of the log χ'' — log ω graph changes from a
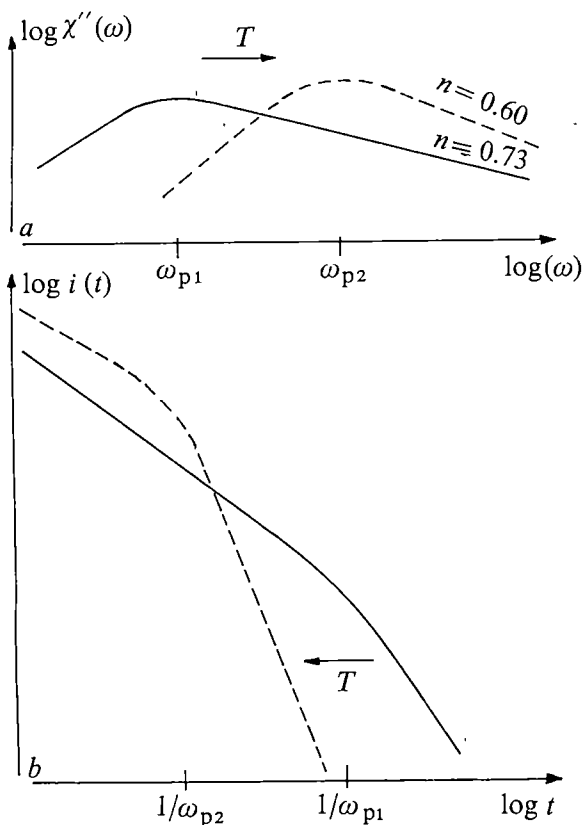
Fig. 2 Schematic representation of the effect of a rise in temperature: a, on $\chi''\omega$, and b, on the corresponding $i(t)$ The slopes m and $(1-n)$ in a reflect qualitatively the observed behaviour. Solid lines, lower temperature. dashed lines, higher temperature.

value of less than unity to one of more than unity; that is, at the 'kink' in the response curve. Similarly, for the Debye mechanism the peak frequency is associated with the time, $t = \tau$, at which the slope goes through -1. But whereas the Debye exponential relationship (equation (3)) gives a smoothly varying slope corresponding to a single physical process in which the polarisation $P(t)$ tends exponentially to the limiting steady-state value, P_0, the empirical relationship is clearly seen to correspond to distinct physical processes. This implies that the polarisation process occurs in two separate stages with transition at a time $t = 1/\omega_p$.

I suggest the following physical significance of these two stages in terms of the mechanism characterised by the two points already mentioned. At time $t < 1/\omega_p$ after the application of the step function field the response $i(t) \propto t^{-n}$ is determined by a 'primary' adjustment of dipoles or hopping charges to the prevailing field, accompanied by the corresponding delayed screening processes. At time $t = 1/\omega_p$ the primary adjustments have been substantially completed and a 'secondary' process sets in, giving the final adjustment of screening and producing the observed, more rapid rate of change of current at times in excess of $1/\omega_p$.

The loss peak in the frequency domain is, therefore, seen as a manifestation of the onset of the secondary processes in the time-domain response which are implicit in the non-Debye model. The temperature dependence of ω_p arises from a shift of the demarcation time towards smaller values with increasing temperature, which may be seen as a result of the primary processes occurring more rapidly. The secondary processes also become more rapid, leading to the observed higher values of the exponent, m, in equation $(1)^{1,2}$ as shown in Fig. 2.

I conclude that the loss peak in solid dielectrics arises from an interaction between two separate processes, which may be described as the primary and secondary relaxations in an

interactive assembly. In this interpretation it seems that the determining factor is the duration of the primary process, after which time the secondary process takes over. This time is thermally activated with a well defined energy and so, therefore, is the peak frequency, ω_p^2.

It is not surprising that the loss peak is not caused by a single 'homogeneous' process as in the Debye model. Even in the accepted interpretation through $g(\tau)$ it is the shape of the distribution function and not the frequency dependence of any particular Debye process that determines the loss peak. The physical significance of $g(\tau)$ has never been made very clear but there is no reason to assume that the rising and falling parts of it are determined by a single physical process.

The foregoing discussion is essentially qualitative and its main object is to point towards a new approach to the old standing and unresolved problems of dielectric relaxation. The justification for this qualitative approach is twofold: the very generality of the phenomena that must be interpreted and the lack of rigour in the accepted point of view. There now remain the difficult tasks of developing a more quantitative approach and also of finding critical experimental evidence against which to test the proposed theory.

I thank S. Bozdemir who performed the numerical Fourier transformations of Fig. 1.

A. K. JONSCHER

*Chelsea College,
University of London,
Pulton Place,
London SW6 5PR, UK*

1 Jonscher, A. K , *Nature*, 253, 717–719 (1975)
2 Jonscher, A. K., *Colloid Polym Sci.*, 253, 231–250 (1975)
3 Jonscher A K , in *Dielectric Materials, Measurements and Applications*, 87–90 (IEE Conference Publication No. 129, 1975)
4 McCrum, N., Read, B E , and Williams, G , *Anelastic and Dielectric Effects in Polymeric Solids* (Wiley, New York, 1967).
5 Lauritzen, J. I , *J chem. Phys.*, 28, 118 (1958).
6 Stoll, B , Pechhold, W , and Blazenbrey, S., *Kolloid-Z. Z. Polymere*, 250, 1,111–1,130 (1972)

Evolution of Proterozoic basement patterns in the Lewisian Complex

Two contrasting structural elements, both of which have affinities with the two Proterozoic mobile belts of Greenland, have contributed to the pattern of structures in the Lewisian complex of the northern British Isles. The evolution of these structural patterns can be interpreted in terms of an integral system of movements which developed under the influence of an evolving linear dislocation that flanked the major Archaean craton of Greenland.

Allowing for continental drift, the Precambrian basement terrains of the North Atlantic Province (Greenland, Labrador and north-western Britain) outline an Archaean craton about 600 km across, which became stabilised about 2,500 Myr ago. Two major Proterozoic mobile belts apparently related to others in the neighbouring Canadian province, border this North Atlantic craton (see ref. 1) to the north, the Nagssugtoqidian, characterised by crustal shortening perpendicular to the belt[2], high pressure regional metamorphism and limited igneous activity, and to the south, the Ketilidian, in which vertical or transcurrent displacements, low pressure metamorphism and widespread magmatic activity occurred The continuations of these belts may intersect in the Lewisian of north-western Britain[2]. The Lewisian comprises a number of small stable blocks of the order of 10 km across composed of Archaean rocks showing relatively slight, late modifications (Scourian complexes) set

in a framework of Proterozoic structures (Laxfordian complexes). North-westerly and north-easterly trending structures within this framework impart the characteristic Proterozoic tectonic 'grain' to the Lewisian, although north-easterly trending Proterozoic structures have only been described in northern Lewis[3].

The occurrence of linear structures along the margins of regions which have been little affected by Proterozoic reworking is a characteristic of north-westerly trending Proterozoic belts in the Lewisian Using Archaean basic igneous complexes as marker horizons, it is possible to identify large-scale isoclinal folds with north-westerly trending axial surfaces, which have existed in the region south of Loch Laxford since at least the end of Archaean granulite facies metamorphism (about 2,800 Myr ago) Other north-westerly trending structures, such as those of the Ness region, Lewis, also seem to have begun to develop in pre-Laxfordian times[3,5]. This suggests that Lewisian structures with north-westerly trends have evolved on, or are related intimately to, a series of lineaments which originated during the late Archaean. Discounting local variations in finite strains, both the Laxford and Ness regions were sites where strain trajectories in the *xy* plane (axial surfaces and schistosity) had gentle to moderate dips and where displacements occurred obliquely to, but effectively perpendicular to, the north-westerly trending lineaments immediately before and after the emplacement of the dolerite Scourie Dykes[7]. At Laxford, movements on shear zones seem to have resulted in local crustal shortening by the overthrusting of Scourian granulites on to remobilised migmatites[8]. Throughout much of the Lewisian of the mainland and the Isle of Lewis, the regions occupied by flat lying, early Laxfordian structures and tectonite fabrics have suffered moderate to high early Laxfordian strains which were apparently caused by large scale, horizontal, ductile shearing

By late Laxfordian times, however, the Ness region was the site of steep *xy*-plane strain trajectories, suggesting displacements around the margins of a more competent block of central Lewis[7]. Similarly, at Laxford, a zone occupied by late Laxfordian granite sheets developed as a steep shear zone[8], parallel to the north-westerly trending lineament.

A north-easterly trending zone of steep dipping foliations lies along western Lewis and intersects the north-westerly trending structures of Ness, in Lewis. This steep zone was probably in existence in pre-Laxfordian times[3] and persisted as a discrete zone of steep foliation compatible with displacements parallel to the margin of the lineament into the late Laxfordian[7]. A similar north-easterly trend has been recognised on geophysical evidence, and is attributed to variations in rock densities, which originated in pre-Laxfordian times[9]; it is also associated with the widespread occurrence of successive groups of late Laxfordian granitic intrusions along the western seaboards of Harris and Lewis.

This arrangement of structures suggests that two systems with contrasting strain trajectories and contrasting major displacements, have acted contemporaneously throughout much of the Proterozoic. Although most influence seems to have been exerted by the zones within which deformation with shallow-dipping *xy*-plane trajectories and displacements mainly perpendicular to the north-westerly trending lineaments occurred during the early Proterozoic, steeply-dipping *xy*-plane trajectories and displacements parallel to the strike were established in the north-easterly trending structure at the same time as displacements on shallow dipping movement planes occurred. Finally, steeply-dipping *xy*-plane trajectories were established parallel to the lineaments in both north-easterly and north-westerly trending structures. Most probably, major discrete dislocations which behaved in a more ductile manner and which separated less ductile 'stabilised' regions, became

more influential during the later stages of Proterozoic evolution in both the Lewisian[3] and in eastern Greenland[15].

Mineral parageneses indicate that a change occurred from the high pressure environment that existed in the Scourian and early Laxfordian[10], to a low pressure environment during the mid to late Laxfordian[11,12]. This off-loading of confining pressure was probably connected with the change from the sub-horizontal crustal shortening (conceivably entailing at least local crustal thickening) that characterised the early Proterozoic to the sub-vertical zones of displacement that dominated the late Proterozoic.

Although it is clear that no single directional element in the tectonic pattern of the Lewisian could be compared directly in scale or behaviour with any individual belt of Greenland, it seems that the Proterozoic patterns in the Lewisian complex are the results of the integration of two nearly contemporaneous but contrasting evolutionary processes somewhat resembling those recorded in separate parts of the adjacent area of Greenland.

In the Lewisian, the north-westerly trending lineaments are distinct sites within which metamorphism and magmatic activity were often directly related to tectonic movements[16], whereas in the north-easterly trending lineaments metamorphism and magmatic activity seem only to be related in a general way to tectonic activity[3,9]. The relatively large, gently dipping zones within which ductile shearing occurred perpendicular to the lineaments under high pressure and temperature conditions during the early stages of development of the Lewisian belts can loosely be compared with the dominant displacements in the Nagssugtoqidian mobile belt[2]. In contrast, the zones of near vertical displacements, relatively low confining pressures and successive granite intrusion, which occurred in both regions during the late Laxfordian could be compared with the dominant regime of the Ketilidian mobile belt.

Since sets of structures compatible with both the major belts of Greenland intersect in the Lewisian, it may be that the two major belts of Greenland simply cross in the Lewisian[2]. But although the Ketilidian seems to be part of a major belt of Proterozoic reworking extending from the south-western United States, around the Canadian province, to the Urals[14], there is little evidence of any continuation of the Nagssugtoqidian Belt (Fig. 1). Alternatively, both the

Fig. 1 The Lewisian in the context of Proterozoic structures from Labrador to Scandinavia. *a*, Archaean craton; *b*, Proterozoic lineament; *c*, tectonic trend lines; *d*, regions of Proterozoic magmatism; *e*, Palaeozoic mobile belts; *f*, Palaeozoic orogenic front.
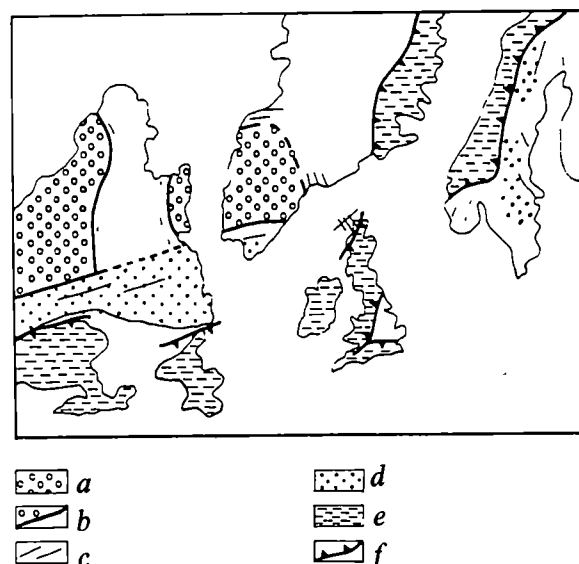
Greenland belts could have suffered displacements similar to those undergone in the Lewisian and it could be that only the dominant set in each belt has so far been recognised. Evidence which might support this suggestion comes from the intersections between tectonic trends in the mobile belts and major dislocation lineaments which border the cratons of Canada, and indicate a sense of movement on major dislocations similar to those in compatible dislocations in the Lewisian[13]. This interpretation of systems of major dislocations indicates the significance of the directional elements in the Lewisian Complex. The evolutionary aspects become clarified if the system of Lewisian structures comparable with the dislocations of the Nagssugtoqidian regime are regarded as relatively early elements in an integral system related to, if not initiated by, movements along steep dislocations associated with a major continuation of the Ketilidian Belt which eventually dominated the part of the craton which is now the Lewisian.

I thank the Inner London Education Authority for a Research Fellowship, and Professor J. Watson and Dr B. F. Windley who read the manuscript.

F. Bryan Davies

Department of Geology,
City of London Polytechnic,
London EC3, UK

1 Bridgwater, D., Watson, J. V, and Windley, B. F, *Phil Trans R. Soc*, A273, 493–512 (1973)
2 Bridgwater, D., Escher, A, and Watterson, J., *Phil. Trans. R Soc*, A273, 513–533 (1973).
3 Davies, F B., Lisle, R J, and Watson, J. V., *Proc Geol. Ass* (in the press)
4 Davies, F B, *J geol Soc Lond*, 130, 279–284 (1975)
5 Evans, C. R, and Lambert, R. St J, *J geol Soc Lond*, 130, 125–150 (1974)
6 Beach, A., Coward, M P, and Graham, R., *Scott J Geol*, 9(4), 297–380 (1974)
7 Davies, F. B, thesis, Univ. London (1974)
8 Beach, A, *Proc Geol Ass*, 85 (1), 13–21 (1974)
9 McQuillan, R., and Watson, J. V, *Nature phys Sci*, 245, 1–3 (1974)
10 Dearnley, R, *Q Jl geol Soc Lond*, 118, 143–176 (1962)
11 Davidson, C. F, *Trans R Soc Edinb*, 61, 71 (1943)
12 Coward, M P., Francis, P. W., Graham, R, Myers, J S., and Watson, J. V., *Proc. Geol Ass*, 80, 387–409 (1969).
13 Sutton, J, and Watson, J. V, *Nature*, 247, 433–435 (1974)
14 Bridgwater, D., and Windley, B. F, *Spec Publs geol. Surv. Un S Afr*, 3 (1973)
15 Escher, A, Watterson, J., and Jacks, S, *Phil Trans. R. Soc.* (in the press)
16 Sutton, J, and Watson, J V., *Q Jl geol Soc Lond*, 106, 251–307 (1951)

A psychological analysis of observation in geology

ANALYTICAL methods of experimental psychology applied to observations of geological data reveal that what geologists perceive in, and remember of, rocks is not necessarily the same as what is actually there. For example, when observing folds, professionals give more attention to antiforms than synforms and tend to remember cleavage fans as they ought to be rather than as they are.

Geology is a science which relies very heavily, at the stage of data collection, on the sense of sight. Experimental methods, which allow an assessment of common biases in perceiving and memorising can show where presented evidence could be distorted. Standardised observation tests are also valuable for training because they provide accurate feedback. This is not always obtained easily during actual geological work because 'the solution' is itself what is sought—it is not known beforehand

To avoid continually concentrating on biases in observation the investigation reported here was also directed towards finding evidence of perceptual attributes so that these, if present, can be used more widely.

For standardisation I constructed stimulus items of controlled content and complexity In order, however, for the geologists to construe the items as symbolic of rocks, they were not drawn too schematically.

To examine possible location and shape biases in the visual assessment of folds, 22 geologists and 22 non-geologists were individually shown Fig. 1a They were instructed to look at the picture and, at 10 random moments chosen by the experimenter, to indicate, by pointing, where they were looking. The indicated points were then recorded on an outline copy of the original Figure 1b shows the synopsis of plots from both groups of subjects and Fig. 1c shows the data contoured.

Several points emerge clearly from this simple experiment. First there are far more plots in antiforms than synforms $(0.001 > P > 0.0001)$, more in hinges than limbs and more in the black buckled layers than in the sandwiched, less competent layers (an average of 50 compared with 36). There are also more plots in the upper than in the lower half of the scene (191 plots compared with 133, central layer data neglected). Non-geologists differed markedly from geologists in having more plots in the upper halves of layers than in the lower halves (irrespective of whether antiforms or synforms are considered). Within the upper layer the difference in plots for non-geologists is at the 0.1% level and for the lower layer $P = 0.05$; (for geologists $P > 0.25$ for both). This accords with the introspections of the control group—many saying that they tended preferentially to scan the upper boundaries Non-geologists also gave less attention to the (what is to a geologist) unusual fold at the left in the upper thin layer (see Fig 1b and c).

There was no tendency in either group for there to be more concentration on the outer arcs of folds rather than the inner arcs $(P > 0.25$ for both groups).

Fig. 1 Geologists tend to describe (a) as comparing fairly tight angular upright folds and (d) as comprising well rounded fairly open folds. Yet (d) is merely (a) rotated through 180°. b, Plots of location-of-gaze (i, 22 geologists; ii, 22 non-geologists); c contoured (at 1, 1 5, 2 and >2% intervals) data from (b) (i, 22 geologists; ii, 22 non-geologists).

a

b

c

d

e
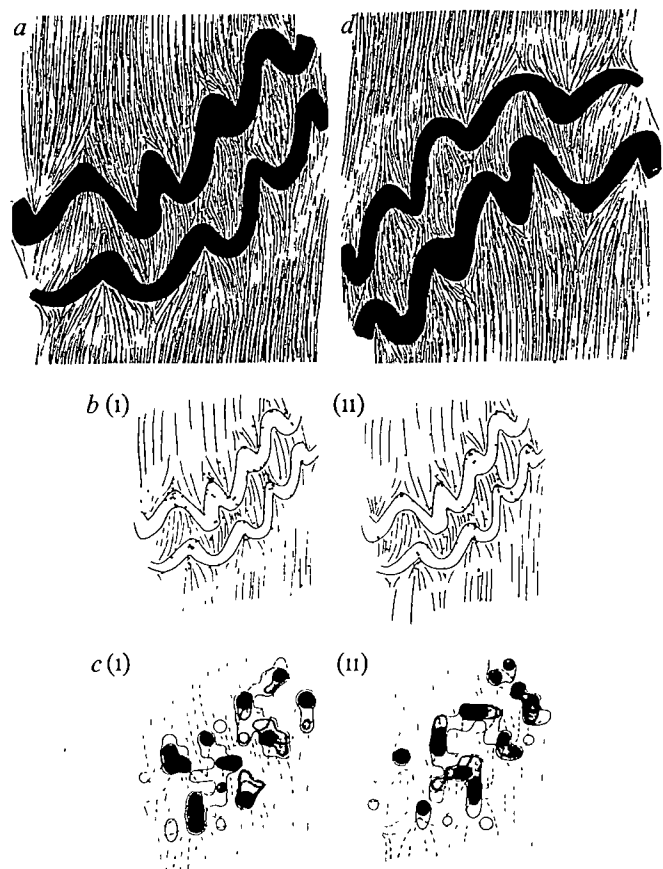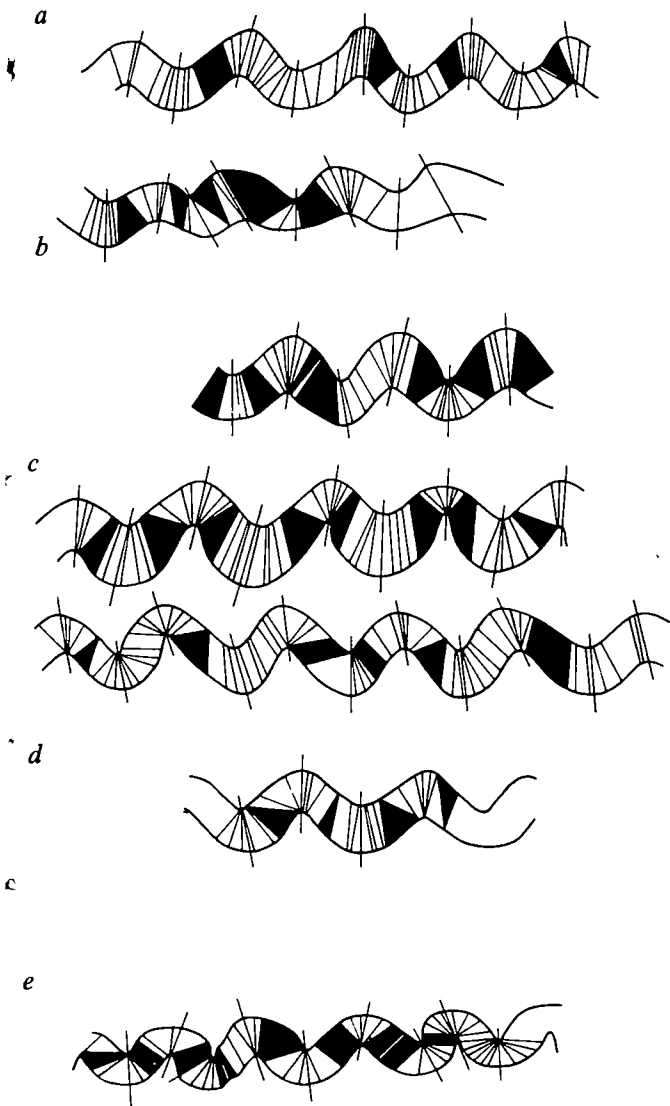
Fig. 2 Isogons (lines connecting points at which interface dips are equal) drawn on folds in which the amplitude and arc length of deflection of the inner and outer arcs is unequal. *a–e*, Five randomly selected portions of fold trains of buckled sandy layers in silt (Ordovician, Hommelvik-Hell region, Norway) Black areas, 'zones of no plots' where no isogons are possible.

It seems that irrespective of the speciality of the geologist the buckled thinner layers tend to be perceived as antiform 'chunks' joined at synform hinges, not the converse (compare Fig. 1*a* with Fig 1*d*).

The preference towards selecting antiforms may be reflected neurologically in a biasing at the level of complex cells in the occipital cortex where there is sensitivity to both orientation and spatial location. Alternatively, one could conceive of the preference for antiforms as resulting from a tendency to look at the upper halves of many objects in everyday life in order to extract the maximum amount of most useful information in the shortest time. That strategy may be transferred to scientific work—in this case to the observation of fold pairs—leading to differential visual scrutiny The experiment indicates that when geologists report, say, that "the most common F₂ folds" in an area are of a certain type, they may, to a large extent, be reporting what are really the most common antiformal folds.

That is a serious bias because antiforms and synforms may differ systematically both in geometry and interior

detail when, for instance, a folded layer is graded or current bedded. It is, therefore, important to remember that synforms are just as important as antiforms and should not be neglected in visual work.

The concentration of visual attention on hinge zones of folds at the expense of limbs, may be one of the reasons why one limitation in Ramsay's classification of folds[1] has been neglected. The use of isogons in the analysis of fold

Fig. 3 Geologists were requested to construct axial surfaces through the folded layers in (*a*) (taken from the Dalradian Ballachulish Limestone on Eilean Munde in Loch Leven, Argyllshire); *b*, combined data from all subjects; *c*, my interpretation (most of the construction lines used to locate hinge points have been left on; axial surfaces dashed).

a

b

c
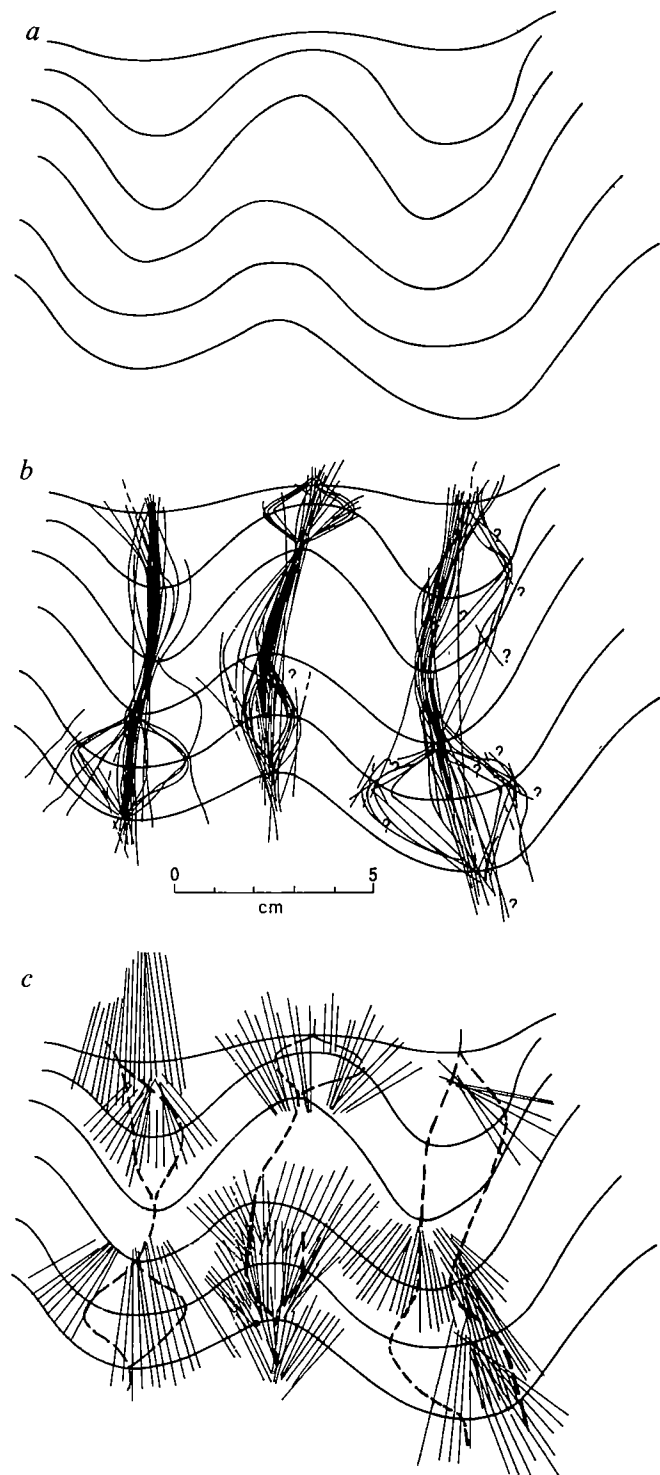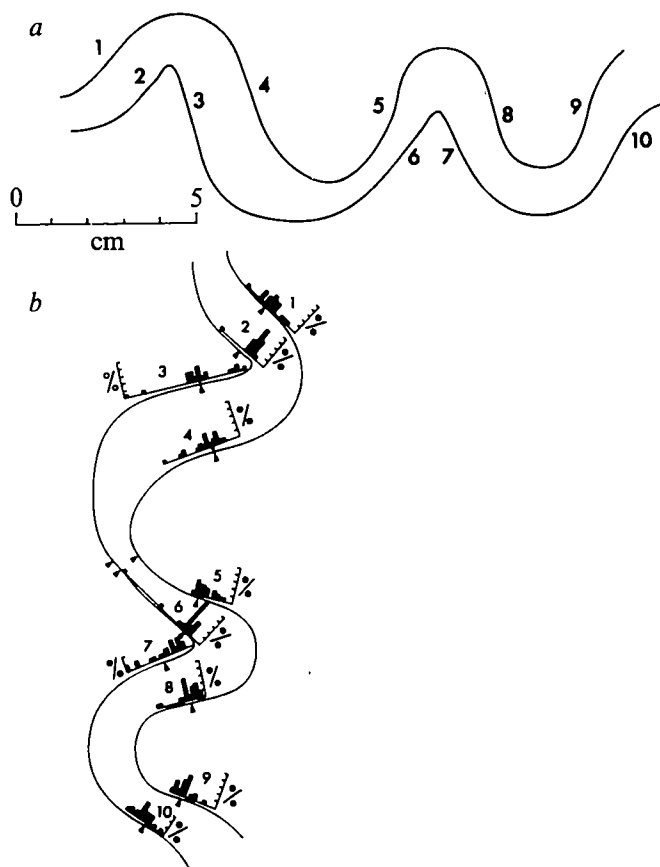
Fig. 4 Geologists were requested to locate inflection points on the limbs of the folds shown above Below are microhistograms (ordinate was 10% divisions) of the locations with data combined from 22 structural geologists Arrows, actual inflection points in the original example.

in Fig. 4. In these moderate and high limb dip-folds at least, the inflection points tended to be located too high up on the limb by 17 of the 22 subjects, irrespective of the dip direction of the limb. The overall mean ratio of up-dip to down-dip locations is 3.25: 1 $(0.001 > P > 0.0001)$ Were such folds being so subdivided for Fourier analysis, it would lead to a spurious increase in synform amplitude and wavelength and a complementary decrease. in those parameters in the antiforms. To compensate for the bias the

Fig. 5 A divergent cleavage, when presented in a buckled layer, is reproduced more frequently when presented in the antiform (*A*), than when in the synform, (*B*), irrespective of whether positioned on the left or the right in the visual field. The object was a slide stimulus projected so as to measure about 2 m × 2 m. Subjects were requested to reproduce the item by freehand drawing immediately after stimulus offset, and (*C*) shows the percentage of subjects who remembered the presence of the divergent cleavage in the folded layer (cross-hatching, when the cleavage occurred in the antiforms; criss-crossing, in the synforms). Subjects were tested in groups: *a*, inexperienced and pre-university; *b*, 85 subjects who were beginning geology, were university undergraduates or were 'O' or 'A' level pupils; *c.* 77 subjects with 1–2 years' university experience, *d*, 6 subjects with between 2 and 36 years' experience (Note that in this case experience is correlated with age)

geometry can only be fully applied if the limb dip at the inflection points of the inner and outer arcs is identical To the unaided eye, they seem to be closely the same in strongly flattened folds but at lower shortenings there is usually a portion of the limb which clearly cannot be analysed because of the differential dip of the two layer surfaces In Fig 2 the black areas are those in which isogons cannot be constructed and orthogonal thickness cannot be measured. Differences in amplitude and arc length between inner and outer arc and differences in shape between antiforms and synforms are inevitable in natural folds because the resistance on one side of a layer will never be exactly the same as the resistance to growth in the opposite half space The differences would be even greater if the layer were graded.

Thirty structural geologists were requested to construct axial surfaces through the folded laminations shown in Fig. 3*a*; a synopsis of the constructions from all subjects is shown in Fig 3*b*. with my interpretation constructed on an enlargement (× 3) (Fig. 3*c*) Apart from the expected variability in the constructions, 60% of the subjects tended, in this 'open ended' task, to avoid any construction of bifurcating axial surfaces. Only 12.5% drew all three surfaces as bifurcating in at least some places. This is an omission in analysis which could lead to an inordinately low impression of the prevalence of conjugate and box-fold geometries in naturally deformed rocks. This neglect of multiple-hinged folds is also reflected by the fact that most geologists do not expect conjugate folds to be present on a mappable scale

Twenty-two structural geologists were requested to locate inflection points on the folded layer interfaces shown

Fig. 6 *A*, Percentage of cleavage reproductions that were shown. as symmetrical from synform to antiform by subjects requested to reproduce the asymmetrical cleavage patterns shown in (*B*). *a*, 53 inexperienced subjects; *b*, 31 'O' level pupils; *c*, 54 subjects with 1–2 years' experience; *d*, 41 subjects with more than 2 years' experience. *C*, Common reproduction of (*B*).

inflection points could be located twice with the fold train rotated through 180° for the second analysis. In those cases where the two points are not identically placed a mid-point between them could be used.

Accuracy of recall from visual short term memory is necessary in observation work; for instance, in making field notebook drawings. To examine such recall, 300 subjects with experience of geology ranging from 2 weeks up to 36 years, in groups ranging from 6 to 50 in number, were shown a randomised sequence of 10 slides which represented varied cleavage fan geometries in folds of single layers. The subjects were required immediately to draw each of the cleavages from memory after viewing the slide stimulus
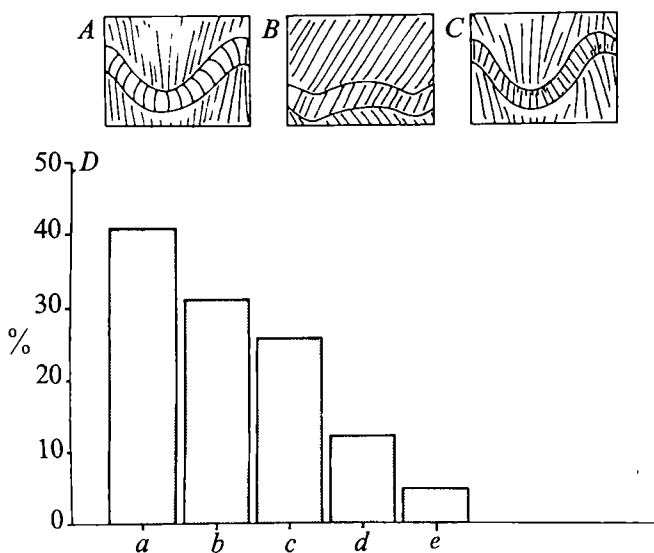
Fig. 7 Subjects were shown *A*, *B* and *C*, and asked to reproduce them, *D*, percentage of subjects who incorrectly reproduced the layer cleavage in (*C*) as curved: *a*, 44 subjects; *b*, 32 'O' Level pupils, *c*, 40 'A' level and first year university students; *d*, 51 subjects with more than 1–2 years' experience; *e*, 40 subjects with more than 2 years' experience.

for either 7 s or 10 s, depending on the slide. This relatively short viewing time was given because of the limited detail in each slide.

The first conclusion from this experiment is that reproductions of antiformal cleavage fans are much better than those for synformal cleavage fans (Fig. 5). The bias towards better antiform reproduction does not decrease with experience (a divergent cleavage—in the buckled more competent layer—was used here as a stimulus item because it was never, in any of my previous experiments, wrongly reproduced as a guess by any subject when the stimulus item did not contain it). Cleavage fans that were asymmetrical from antiform to synform (Fig. 6), as could occur, for instance, in a folded, graded layer, tended to be reproduced, if at all, as symmetrical from antiform to synform by about 20% of subjects in each experience group.

In some groups the first slide presented had curved cleavage traces and the third was similar to it in many respects, except for the fact that the cleavage traces were straight. The percentage of subjects who misremembered the cleavage in the third slide as curved (that is, the percentage who transposed detail from the first to the third item) did decrease as a function of experience (Fig. 7). On the other hand, cleavage traces which were convergent in the matrix inside the inner arc of the buckled layer were more often misremembered by professional structural geologists as divergent. This is the way matrix cleavage fans are usually represented in text books of structural geology and also, presumably, in the long term memories of the structural geologists.

It was found that the way the object on the screen was construed by subjects could vary as a function of bias introduced by the experimenter and that this could influence the scanning and search strategies used For instance, a palaeontologist who came into a laboratory during the display of one of the sequences (and who had therefore missed the initial instructions) thought that the so-called folds and cleavages were worm-tubes and disrupted laminations. On the basis of introspection he detected no evidence of antiform preference in himself. Rather than looking mainly at th antiforms and. by redundancy, assuming the synform to be symmetrical, he instead scanned along the 'tube' and therefore recognised the differences between the antiforms and the synforms.

P. K. CHADWICK*

Institute of Geology,
Uppsala, Sweden

1 Ramsay, J G., *Folding and fracturing of rocks* (McGraw Hill, New York, 1967)

Exercise and human sleep

IT has been proposed that slow wave sleep (SWS) in humans is associated with somatic restitution[1], and findings of increased plasma somatotrophin during SWS[2], and of the greater amounts of SWS following exercise[3,4] support this proposal. Others have, however, failed to show any exercise effects of sleep[5,6]. Exercise has been found to increase sleep somatotrophin levels, but not SWS[7]. These contradictory findings may in part be attributed to the large interstudy variations of administered exercise and fitness of subjects (in no study has exercise been standardised against individual work capacity); the time of day of exercise; the need for further qualification of SWS, defined[8] here as sleep stages 3 and 4, respectively containing 20–50% by time of delta activity, and more than 50% delta activity. Up to 20% delta activity is included in the non-SWS stage 2. We have investigated the effects of the time

Table 1 Group means (%) for sleep stages

	Baseline	Morning exercise		Afternoon exercise	
		Recovery night	Carryover night	Recovery night	Carryover night
Whole night					
Stages 0 + 1	5 2	6 2	5.7	5.4	4.8
2ii	9.9	8.8	8.9	11.7	9.6
3	9.5	9.7	10.8	10.8	8.5
4	18.2	19.1	17.2	17.2	16.8
3 + 4	27.7	28.8	27.9	27.9	25.3
2ii + 3 + 4	37 6	37 6	36 8	39.6	34.9
REM	23 5	25.2	24.1	23.7	24.4
First half					
Stages 0 + 1	2 8	3.2	3.8	2.7	2 6
2ii	11.6	8 0†	10.0	14.6	12.2
3	13.2	11.5	13.6	16.2†	11.0
4	31.6	35.3	26.1	29.3	30.8
3 + 4	45.1	46.7	39.7	45.3	41.9
2ii + 3 + 4	56.7	54.7	49.7*	59.9	54.1
REM	13.7	15.9	16.3	13.2	14.5
Second half					
Stages 0 + 1	7.8	9.2	7.6	8.2	7.1
2ii	8.2	9.5	8.1	8.8	7.1
3	5.7	8.0	7.9	5.3	6.1
4	4.9	2.8	8.2	5.1	2.8
3 + 4	10.4	10.9	16.1	10.8	8.7
2ii + 3 + 4	18.6	20.4	24.3	19 6	15.8
REM	33.3	34.4	31.8	34.7	34.4

*Significant at 0.05 level (two-tailed).
†Significant at 0 01 level (two-tailed).

of day of individually standardised amounts of exercise on subsequent sleep in which stage 2 was subdivided further between 10% and 20% delta activity (2ii).

Eight healthy male subjects (18–22 yr), of average build and fitness, were assessed for individual maximum aerobic power during steady-state exercise on a bicycle ergometer by the Nomogram method[9]. From the maximum work capacity calculated, each subject was prescribed a workload 45% of this capacity. It was further established that this load could be maintained without undue stress for 85 min, with a 15 min break at the half way point. In the main study the exercise was performed by each subject on two separate days, once in the morning, and once in the afternoon. Electroencephalograms were obtained throughout the sleeping period for each exercise night, and for a following 'carryover' night. There were initial adaptation nights and two baseline nights. Subjects were measured in pairs with one performing morning exercise and the other evening exercise on any one day. Diet, naps, extraneous exercise and alcohol intake were controlled. Sleep records were independently scored into the recognised sleep stages[8] and stage 2ii. Group data were compiled for the sleep variables shown in Tables 1 and 2. Related t tests were used to compare the data for each of the experimental nights with the average baseline values.

Tables 1 and 2 show that there are few significant findings. Whole-night percentages of each sleep stage and parameter,

including 2ii, during all experimental nights remained within baseline limits. During the first half of the afternoon exercise night, and particularly before the first rapid eye movement (REM) period, stage 3 showed a significant increase. Although stages 4 and 2ii before the first REM period tended to show increases, they did not individually reach significance. On this night three subjects missed their normally expected first REM period, producing an unusually long delay to a first REM period and enabling more SWS to occur before the first REM period. These three REM period delays seem to be mainly responsible for the longer, but non-significant, REM latency. There are no significant effects for any parameter during the afternoon exercise carryover night. Stage 2ii shows two related significant decreases on the morning exercise night, one before the first REM period and the other for the first half of the night. Any possible loss of delta activity here, however, may have been made up in the slight increase of stage 4, the more substantial delta activity sleep, during the first half of the night. On the morning exercise carryover night, summated delta activity stages 2ii+3+4 showed a significant decrease during the first half of the night, which seems to be reciprocated by a significant increase during the second half. This seems to be a real temporal displacement, rather than an artefact of the division line of sleep into halves. The reason for this is not known.

Our findings seem to show a time of day effect of exercise on

Table 2 Group means for REM parameters

	Baseline	Morning exercise		Afternoon exercise	
Time (min) before first REM period		Recovery night	Carryover night	Recovery night	Carryover night
Stages 0 + 1	1.0	0.7	1.4	2.1	1.7
2ii	10.6	5.4*	7.8	16.6	9.3
3	14.4	10.7	12.5	19.6*	11 6
4	47.9	50.2	42.0	56.2	40.0
3 + 4	62.1	60 9	54.5	75 7	51.6
2ii + 3 + 4	72.7	66 3	62.3	92.3*	60 9
REM latency	91.9	79.4	79.4	112.9	89.5
First REM period length	14 4	11.9	9.0	15.8	10 9
REM periodicity					
First period	88.4	87.9	77.2	77.1	81.7
Mean of first and second periods	92.3	92.0	90.4	89.9	89.6

*Significant at 0.05 level (two-tailed).

recovery sleep. The main SWS delta activity increase was for stage 3 during the first half of the night following afternoon exercise, and this seems to be partially counterbalanced by a non-significant decrease during the second half of the night, resulting in no significant overall changes. If SWS reflects enhanced protein synthesis and body restitution[1], then in view of the high workload imposed, more substantial changes in SWS might have been expected. It must be concluded that if a heavy, but tolerable, workload is imposed early in the day then ensuing wakefulness is sufficient for recovery. If this exercise is given later in the day, however, then ensuing wakefulness may not be adequate for recovery, and some of the recovery process may intrude into the earlier part of sleep and be reflected in the sleep EEG.

J. A. HORNE
J. M. PORTER

Department of Human Sciences,
Loughborough University,
Loughborough,
Leicestershire, UK

[1] Oswald, I., *Nature*, **223**, 893 (1969).
[2] Sassin, J. F., *et al.*, *Science*, **165**, 1299 (1969).
[3] Baekeland, F., and Lasky, R., *Percept. Mot. Skills*, **23**, 1203 (1966).
[4] Zloty, R. B., Burdick, J. A., and Adamson, J. D., *Activ. nerv. sup. (Praha)*, **15**, 217 (1973).
[5] Hauri, P., *Psychophysiology*, **4**, 267 (1968).
[6] Zir, L. M., Smith, R. A., and Parker, D. C., *J. clin. Endocr.*, **32**, 662 (1971).
[7] Adamson, L., Hunter, W. M., Ogunremi, O. O., Oswald, I., and Percy-Robb, I. W., *J. Endocr.*, **62**, 473 (1974).
[8] Rechtschaffen, A., and Kales, A., *A Manual of Standardised Terminology, Techniques and Scoring System for Sleep Stages of Human Subjects* (US natn. Inst. Hlth, No. 204, 1968).
[9] Astrand, I., *Acta physiol. scand.*, **49**, suppl., 169 (1960).

Dihydroactinidiolide in the supracaudal scent gland secretion of the red fox

IN spite of growing interest in the function of skin glands in mammalian chemical communication[1,2], little is known of either the chemical nature or the behavioural significance of the secretion of the supracaudal (tail) scent gland of the red fox (*Vulpes vulpes*). A recent paper[3] reports observations concerning the histology and histochemistry of this gland, including findings of unusual fluorescent sebum constituents. In this paper, preliminary gas chromatographic–mass spectrometric data are presented indicating the presence of dihydroactinidiolide (I) and related compounds in this secretion and the significance of this finding is discussed.

I   II   III
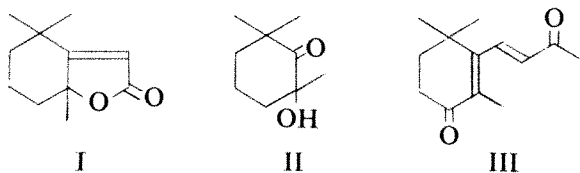
Particles of waxy yellow-brown stale secretion were separated manually from the hair and skin surface of the supracaudal gland region of eleven male red fox (taken wild, February 14, 1974–April 19, 1974, Wales), bulked (67 mg) and distilled (at 170 °C, air bath temperature, 0.02 mmHg). The distillate was examined by combined gas chromatography–mass spectrometry (AEI MS30 mass spectrometer (24 eV) with a Pye-Unicam 104 gas chromatograph fitted with a 2 m × 2 mm internal diameter glass column of 2% SE-33 on 80–100 mesh Gas Chrom Q; column temperature 116 °C to 280 °C at 5 °C min⁻¹; injector temperature 170 °C; separator temperature 200 °C) following treatment with diazomethane.

A number of volatile components exhibiting mass spectra suggesting monoterpenes or related compounds were observed

in advance of the range of saturated carboxylic acids (starting at C-14, observed as methyl esters) and related compounds expected in a distillate of skin lipids. These more volatile compounds included substances possessing the following observed mass spectra (five most intense ions, plus other significant ions) indicating dihydroactinidiolide (I)[4] *m/e* 180 (M⁺, 22), 165 (7), 152 (11), 137 (30), 111 (100), 109 (30), 43 (25); 6-hydroxy-2,2,6-trimethylcyclohexanone (II)[5] *m/e* 156 (M⁺,6), 128 (31), 110 (35), 95 (39), 71 (100), 58 (34), 43 (36); and *trans*-4-keto-β-ionone (III)[6] *m/e* 206 (M⁺,47), 191 (15), 177 (6), 164 (38), 163 (9†), 121 (53), 43 (100), 41 (38). Other major volatile terpenoid constituents of the secretion distillate are under investigation.

The identity of dihydroactinidiolide was confirmed by gas chromatographic coinjection with authentic material, m.p. 42–43 °C (retention time 4.1 min, 1.5 m × 2 mm internal diameter glass column of 5% Dexsil 300-GC on 80–100 mesh Chromosorb W, AW; column temperature 172 °C, isothermal). A peak of the same retention time was also noted when a thin-layer chromatography fraction (acetone pre-eluted silica gel G (Merck type 60), R_f 0.06 to 0.2 with diethyl ether–petroleum spirit (60–80 °C), 30/70, v/v) of a methanol–chloroform extract of stale secretion was examined by gas chromatography (dihydroactinidiolide, R_f 0.08). The level of dihydroactinidiolide in this sample of stale secretion was < 50 p.p.m.

This is the first report of volatile terpenes in an external mammalian glandular secretion, although it was expected that such compounds might be involved in mammalian communication following the elucidation of the olfactory response of certain Felidae to *cis-trans*-nepetalactone, a major constituent of the essential oil of the catnip plant, *Nepeta cataria*[7]. The early description of the supracaudal gland as the 'violet gland' on account of its odour also suggested such a finding. A number of terpenoid constituents of the plant *Actinidia polygama*, including dihydroactinidiolide, are also reported to have behavioural effects on certain Felidae[8].

The presence of photolabile fluorescent compounds has been reported in the red fox supracaudal gland sebum, and fluorescence and ultraviolet spectra have been described[3]. Field desorption mass spectrometry of the major non-polar fluorescent fraction (Varian CH5D, source temperature 80 °C, emitter current 8 mA) revealed major ions at *m/e* 524, 525, 550 and 552 (no ions in range *m/e* 554–1,200) indicating the presence of compounds in the carotenoid mass range. If carotenoids are present, their occurrence could be related to the presence of volatile terpenes in the secretion[9].

E. S. ALBONE

University of Bristol,
Department of Animal Husbandry,
Langford House, Bristol BS18 7DU, UK

[1] Mykytowycz, R., *Advances in Chemoreception*, **1**, chapter 11 (edit. by Johnston, J. W., Moulton, D. G., and Turk, A.) (Appleton-Century-Crofts, New York, 1970).
[2] Birch, M. C. (ed.), *Pheromones* (North Holland, Amsterdam and London, 1974).
[3] Albone, E. S., and Flood, P. F. (in the press).
[4] Chen, P. H., Kuhn, W. F., Will, F., and Ikeda, R. M., *Org. Mass Spectr.*, **3**, 199–209 (1970).
[5] Müggler-Chavan, F., Viani, R., Bricout, J., Marion, J. P., Mechtler, H., Reymond, D., and Egli, R. H., *Helv. Chim. Acta*, **52**, 549–550 (1969).
[6] Ina, K., and Eto, H., *Agr. Biol. Chem.*, **36**, 1091–1094 (1972).
[7] Todd, N. B., *J. Hered.*, **53**, 54–56 (1962) and refs therein.
[8] Sakan, T., Isoe, S., and Hyeon, S. B., *Tetrahedron Lett.*, 1623–1627 (1967).
[9] Isoe, S., Hyeon, S. B., and Sakan, T., *Tetrahedron Lett.*, 279–281 (1969).

Ethylene in soil

THE origin of ethylene in the soil atmosphere is of interest because the concentrations at which it sometimes occurs can restrict plant growth by inhibiting root extension in cereals[1] and root nodulation in legumes[2]; it has also been suggested that it aids plants by inhibiting pathogenic fungi[3]. Our earlier observations showed that ethylene could be produced by a soil fungus, *Mucor hiemalis*, and two soil yeasts, which have now been identified as *Candida vartiovaarai* and *Trichosporon cutaneum*, when glucose and methionine were available to them[4]. Subsequent investigations in pure culture and in soil have attempted to elucidate mechanisms by which these organisms could synthesise the gas[5-7]. Other investigators[3,8] have proposed that anaerobic spore-forming bacteria rather than fungi are largely responsible for ethylene formation in soil as: (1) heat does not prevent the formation of the gas in soil, (2) anaerobiosis favours its formation and (3) the gas is fungistatic. Further experiments have therefore been undertaken in an attempt to assess if these latter observations cast doubt on our conclusions; the results are reported here.

Using previously described techniques[8] we have confirmed earlier reports that heat[8] and even autoclaving[9] can stimulate the production of ethylene by soils (Table 1). The effect of heat at 100 °C on the release of ethylene from pure cultures of *M. hiemalis* was therefore investigated. No ethylene was produced by heating the culture medium alone but, although the treatment killed the fungus, the evolution of the gas from the treated culture was greatly enhanced compared with that of cultures kept at 25 °C (Fig. 1). Some observations in our earlier work suggest that a possible explanation for this is that *M. hiemalis* forms an intermediate substance in the pathway to ethylene which passes into the extracellular solution and breaks down non-enzymically, a process promoted by heat at 100 °C (ref. 6). Another possible explanation is that heat decreases the growth rate and in the range of growth rates 0.08 h^{-1} to 0.02 h^{-1}, there

Table 1 Effect of heat treatment on ethylene production by two soils

	Ethylene nmol per 100 g soil per 6 d	
	Chalk loam (Gore series)	Basaltic loam (Woodburn series)
Treatment	6.6% organic matter	9.8% organic matter
Boiling (100 °C per 10 min)	8.0	23.4
Autoclaving (121 °C per 60 min)	3.4	7.2
Control (unheated)	0.7	22.9

Soils (5 g) were wetted and incubated in glass vials (14 ml)[8].

is an exponential increase in ethylene production[7]; furthermore the subsequent cell lysis also increases the production of the gas[5].

The production of ethylene after heating soil is therefore consistent with a fungal origin. Moreover we have obtained evidence that although ethylene is normally produced in anaerobic conditions[10], the importance of these conditions seems to lie in the provision of substrates, which we have recently found can be provided by crop residues. When suitable substrates, glucose and methionine, are provided the production of ethylene by *M. hiemalis* and by soil is stimulated by oxygen[5]. Furthermore anaerobiosis can reduce fungal growth rate which increases ethylene production[5,7] and inhibits ethylene breakdown by soil microorganisms[11]. Waterlogging, of which anaerobiosis is a consequence, inhibits gaseous diffusion and retains ethylene in the soil atmosphere.

Fig. 1 Effect of heat (100 °C per 30 min) on ethylene production by pure cultures of *Mucor hiemalis*. The fungus was grown in glucose–methionine medium[5] (100 ml) in flasks (capacity 325 ml) for 48 h to produce 1.5 mg dry weight ml^{-1}. The cultures were then heated and the flasks sealed 30 min after the treatment. The heated medium[5] alone produced no ethylene. ●, Heated; ○, unheated.

Fig. 2 Colonisation of buried straw by a mixed fungal population (identified as *Mucor* sp. and *Fusarium* sp.) in the presence of ethylene; similar fungal development has been observed in the presence of 1,000 p.p.m. ethylene. Saprophytic fungi are the most likely causative agents of ethylene production from organic residues in soil.

If the inhibition of growth by ethylene was a normal characteristic of fungi, they could not be considered as the main producers of the gas in soil. But whereas the pathogenic fungus *Sclerotium rolfsii* is very sensitive to ethylene[3], five other fungal species investigated in this and three other laboratories were found to be relatively unaffected by considerably higher con-

centrations than those found in soil (Table 2). Figure 2 shows the extensive mycelial development which can occur in the presence of high concentrations of the gas and while it might be postulated that ethylene would be fungistatic only in nutrient-limited conditions, we have found little evidence for any soil microbial activity in these conditions.

Table 2 Effect of ethylene on fungal growth			
		Maximum inhibition of growth or germination (%)	
Fungus	Concentration of ethylene (p.p.m.)	In vivo	In vitro
Sclerotium rolfsii[3]	1	100*	—
Fusarium oxysporum[12]	2,000	16†	57
Gloeosporium album[13]	2,000	33†	19
Sclerotinia fructigena[14]	100	22†	12
Mucor hiemalis	1,000	0‡	0§
Aspergillus clavatus	1,000	0‡	—

—, No information.
*Grown in soil.
†On fruit tissue.
‡On decaying barley tissue in soil.
§In 1/100 strength tryptone soya broth.

In our earlier work[4] bacterial isolates from soil produced little or no ethylene whereas fungal isolates produced large amounts. This observation has now been confirmed and in addition studies in pure culture with the ubiquitous soil anaerobe, *Clostridium pasteurianum*, showed negligible production of ethylene. Moreover the only report in the literature on the production of ethylene by bacteria in pure culture relates to *Pseudomonas solanacearum*[15]; in contrast, cultures of over 70 fungi have been shown to produce ethylene[16]. No information now available therefore is inconsistent with saprophytic fungi being responsible for the production of ethylene and in the soils we have studied *M. hiemalis* seems to be a major contributor. This does not prove that anaerobic bacteria do not produce ethylene but indicates that their contribution if any is very minor.

J. M. LYNCH

Agricultural Research Council,
Letcombe Laboratory,
Wantage, Oxfordshire OX12 9JT, UK

1 Smith, K. A., and Russell, R S , *Nature*, 222, 769–771 (1969)
2 Grobbelaar, N., Clarke, B., and Hough, M. C., *Plant and Soil* (special volume), 215–223 (1971).
3 Smith, A M , *Nature*, 246, 311–313 (1973).
4 Lynch, J M , *Nature*, 240, 45–46 (1972).
5 Lynch, J. M., and Harper, S H. T , *J gen Microbiol* , 80, 187–195 (1974).
6 Lynch, J. M., *J. gen. Microbiol.*, 83, 407–411 (1974).
7 Lynch, J M , and Harper, S H. T., *J. gen. Microbiol* , 85, 91–96 (1974)
8 Smith, A M., and Cook, R. J , *Nature*, 252, 703–705 (1974).
9 Rovira, A. D , and Vendrell, M., *Soil Biol Biochem.*, 4, 63–69 (1972).
10 Smith, K A., and Restall, S. W. F , *J Soil Sci.*, 22, 430–443 (1971)
11 Abeles, F. B., Craker, L. E., Forrence, L. E , and Leather, G R., *Science*, 173, 914–916 (1971)
12 Lockhart, C. L , *Can J Pl Sci.*, 50, 347–349 (1970)
13 Lockhart, C. L , Forsyth, F R , and Eaves, C A., *Can. J Pl Sci.*, 48, 557–559 (1969).
14 Hislop, E. C , Hoad, G. V , and Archer, S A , in *Fungal Pathogenicity and the Plant's Response* (edit. by Byrde, R. J W , and Cutting, C. V.), 87–117 (Academic, London, 1973)
15 Freebairn, H. T., and Buddenhagen, I. W , *Nature*, 202, 313–314 (1964)
16 Bird, C. W., and Lynch, J. M , *Chem Soc. Rev.*, 3, 309–328 (1974)

Cytogenetic effects of plutonium-239 in male mice

INCREASING quantities of plutonium-239 are processed in the nuclear power industry. The likely magnitude of the associated genetic risk is still uncertain but thought to be less than the risk of cancer induction[1]. Green *et al.*[2] have recently shown, however, that plutonium reaching the testis after intravenous injection of [239]Pu citrate into CBA mice concentrates in the interstitial tissue, outside the seminiferous tubules. They calculated that the average dose rate to spermatogonial stem cells, in which genetic damage can accumulate, was about 2–2.5 times

that to the whole testis. In these circumstances, the genetically significant dose is higher than the average tissue dose, which is that normally used for protection purposes[1]. To obtain more direct evidence on genetic damage from gonadal plutonium, we are studying the induction of chromosome aberrations in germ cells of male mice after protracted exposure to α particles from [239]Pu. The results of the first experiment, described here, suggest that α radiation has the expected high relative biological effectiveness (RBE)[3] for induction of chromosome damage and that a distribution factor of the magnitude proposed may indeed be operating as well.

Twelve 10-week-old (C3H/HeH♀ × 101/H♂) F₁ male mice were injected intravenously with 10 μCi kg⁻¹ [239]Pu in 1% trisodium citrate solution (about 0.3 μCi per mouse). Three littermate controls were injected with similar quantities of the citrate solution alone. Cages of four treated and one control were kept for 6, 12 and 18 weeks. They were then killed and weighed, as were the testes. Air-dried chromosome preparations[4] were made from cellular suspensions of each right testis, and the left was used for radiochemical assay of plutonium. From each testicular preparation, 100 spermatocytes at diakinesis–metaphase I were scored for chromosome anomalies. Additional procedures on the 18-week batch only were sperm counts from the caput epididymis[5]: examination of 500 spermatozoa from the cauda epididymis for head abnormalities, after staining with eosin Y and making air-dried smears[6]. All slides were coded.

The fraction of injected plutonium retained in the left testis changed very little over the period of the experiment (Table 1); therefore radiation dose was taken to be the product of the assay-derived dose rate (≈ 0.0002 rad min⁻¹) and time. Testis weight declined to 58% of controls after 18 weeks (Table 2). At this time, the mean epididymal sperm count was only 51% of the control value, showing that much of the testis weight loss was the result of germ cell death. The mean frequency of sperm-head abnormalities was 9.0%, compared with 4.6% in controls, but this difference mainly stemmed from one male with 17.2% abnormalities. The causes of these abnormalities are not yet known, but they are thought to indicate genetic damage[6].

In the experimental series, frequencies of autosomal and X–Y chromosome univalents did not differ significantly from control values of $6.0 \pm 1.4\%$ and $12.0 \pm 1.9\%$, respectively. The frequencies of spermatocytes with fragments (Table 2) showed a non-significant increase in the irradiated series; 87% of these fragments were diagnosed as double and thought to be the result of isochromatid breaks. Whereas affected control cells had one fragment each, six cells in the irradiated series had two fragments and three cells had three. Thus the overall fragment frequency after treatment was $3.4 \pm 0.7\%$, compared with the control frequency of $1.3 \pm 0.7\%$ ($P = 0.07$ on one-sided test).

The increase in the frequency of spermatocytes with quadrivalent configurations (Table 2) in the treated series is clearly significant. Most of these were typical rings and chains of four arising from chromosomal reciprocal translocation in spermatogonia, as previously found after X, γ and neutron irradiation of this cell type[7]. A few, however, (especially at the 18-week interval) seemed to resemble more closely the type of quadrivalent previously seen by two of us after X irradiation of

Table 1 Results of radiochemical analysis of left testes of hybrid mice after injection of [239]Pu, with determinations of average radiation dose

Weeks of exposure	No. of mice	[239]Pu in left testis (± s.e)		Dose* (rad)
		% Injected activity	pCi	
0	3	—	—	0
6	4	0.042 ± 0.002	130 ± 9	14
12	4	0.048 ± 0.002	143 ± 13	30
18	4	0.040 ± 0.004	134 ± 28	44

*For calculations of dose, the average of the control and the mean experimental left testis mass for the batch concerned was used, and the mean energy of a single α particle from [239]Pu was taken as 5 15 MeV (ref. 16).

Table 2 Mean testis mass and chromosome aberrations at metaphase I at different times after injection of ^{239}Pu (percentages in brackets)

Weeks of exposure	Testis mass (mg ± s.e.)	Spermatocytes scored	Number with		Reciprocal translocations		
			Fragments	Quadrivalents*	Rings	Chains	Totals
0	125 ± 2	300	4 (1.3)	1 (0.3)	0	0	0 (0.0)
6	91 ± 6	400	8 (2.0)	4 (1.0)	3	1	4 (1.0)
12	88 ± 2	400	9 (2.2)	16 (4.0)	15†	4	19 (4.8)
18	73 ± 8	400	12 (3.0)	9 (2.3)	2	3	5 (1.3)

*Resulting from reciprocal translocations arising in spermatogonia (see next columns) or chromatid interchanges in spermatocytes.
†Three cells each had two rings of four.

oocytes in female mice[8], they were therefore thought to be the result of chromatid interchange in spermatocytes during meiotic prophase.

The yield of quadrivalents was reduced at the longest exposure period, the effect being most marked in the translocation group. A similar decline in translocation frequency has been reported after high acute exposures to X rays[9,10] or fission neutrons[11], but not after protracted exposures to the latter. The same phenomenon occurs with specific locus mutation frequencies after acute exposures[12] and is thought to be connected with the preferential killing of the most mutationally sensitive part of the spermatogonial population by high radiation doses. It is possible that a similar explanation could account for the decrease in the present experiment. Consequently, the low 18-week values have been excluded when determining rates of induction.

The data on reciprocal translocation induction can be used to obtain a rough estimate of the relative effectiveness of the α particles. As these translocations would be expected to arise in spermatogonia rather than spermatocytes (where the chromatid interchange type would be found), the time taken for the germ cells to pass through meiosis to metaphase I (13 d)[13] should be subtracted from the actual exposure period, to give the period of spermatogonial exposure. When these amended doses of 10 and 25 rad are used and linearity of response is assumed the rates of induction of reciprocal translocations at the 6-week and 12-week exposure periods are 1.0×10^{-3} and 1.9×10^{-3} rad^{-1}, respectively, with a mean of 1.45×10^{-3} rad^{-1} of the average testis dose. In previous experiments with the same hybrid male mice, a 600 rad spermatogonial dose of γ rays delivered at 0.02 rad min^{-1} (ref. 14) induced translocations at the rate of 2.3×10^{-5} rad^{-1}, whereas a 62-rad dose of fission neutrons, delivered at 0.0005–0.0008 rad min^{-1} (ref.11) induced them at a rate of 0.53×10^{-3} rad^{-1}. These rates are lower than that estimated for ^{239}Pu α particles by factors of 63 and 2.7, respectively. Thus these results are in good agreement with the assumption that α particles have a similar RBE for translocation induction to that of fission neutrons (previously reported as about 23 for chronic exposures)[15] and that the α-particle dose to spermatogonia is about 2.5 times the mean dose to the whole testis[2]. There are, however, wide error limits to some of these values as well as uncertainty on whether more protracted γ irradiation would have the same effect as the 0.02 rad min^{-1} dose quoted above. Therefore further work is in progress, from which we hope to make a direct comparison of translocation induction by ^{239}Pu α particles and by ^{60}Co γ rays, with similar protracted exposures for both types of radiation.

We thank G. Fisher for assistance with dosimetric aspects, D. Papworth for statistical help and Dr J. Vennart for encouragement and advice.

C. V. BEECHEY
D. GREEN
E. R. HUMPHREYS
A. G. SEARLE

MRC Radiobiology Unit,
Harwell, Didcot,
Oxfordshire OX11 0RD, UK

1 Medical Research Council, *The Toxicity of Plutonium* (HMSO, London, 1975)
2 Green, D., Howells, G. R , Humphreys, E. R , and Vennart, J., *Nature*, 255, 77 (1975).
3 ICRP, *Recommendations* (ICRP publication 9, Pergamon, Oxford, 1966).
4 Evans, E. P., Breckon, G., and Ford, C. E , *Cytogenetics*, 3, 289–294 (1964).
5 Searle, A. G , and Beechey, C. V., *Mutat. Res.* 22, 63–72 (1974).
6 Bruce, W. R., Furrer, R , and Wyrobek, A J., *Mutat Res.*, 23, 381–386 (1974)
7 Léonard, A., *Mutat. Res.*, 11, 71–88 (1971).
8 Searle, A. G., and Beechey, C. V , *Mutat. Res.*, 24, 171–186 (1974).
9 Leonard, A., and Deknudt, G., *Radiat. Res.*, 40, 276–284 (1969).
10 Lyon, M. F , and Morris, T , *Mutat. Res*, 8, 191–198 (1969). ·
11 Searle, A. G., Evans, E. P , and West, B J., *Mutat. Res.*, 7, 235–240 (1969)
12 Russell, W. L., *Genetics*, 41, 658–659 (1956).
13 Oakberg, E. F., *Nature*, 180, 1137–1139 (1957)
14 Searle, A. G., Evans, E. P., Ford, C. E , and West, B J., *Mutat. Res.*, 6, 427–436 (1968).
15 ICRP, *The RBE for High-LET Radiations with respect to Mutagenesis* (ICRP publication 18, Pergamon, Oxford, 1972).
16 Lederer, C. M , Hollander, J. M., and Perlman, I., *Table of Isotopes*, sixth ed. (Wiley, New York, 1967).

Reversible arrest of mouse 3T6 cells in G_2 phase of growth by manipulation of a membrane-mediated G_2 function

BESIDES replicating their genetic material and subsequently partitioning it between two daughters, cells of higher organisms also generally contain two distinct periods during their growth cycle in which they are engaged in neither activity. The first of these is a gap which occurs between the completion of one division and the onset of the next round of DNA synthesis, and is abbreviated as G_1. The ability to obtain stable populations of cells reversibly arrested in G_1 (refs 1–3) has made possible extensive investigation of this stage of the cell cycle[4]. The second gap (G_2) lies between the completion of DNA synthesis and the onset of mitosis. Chiefly because of the unavailability of techniques for reversibly holding cells at this stage, the possible functions of G_2 have remained largely unprobed[5]. I now report a method by which reversibly-arrested G_2 cells can be obtained and tentatively suggest one function of this phase as being the reorganisation of the cell surface in preparation for the start of mitosis.

It has become increasingly evident over the past few years that cell membranes are fluid regions[6–9] within which movements of constituent macromolecules such as surface antigens[10], membrane-bound enzymes[8], and surface agglutinin receptor sites may occur[11,13]. Such movements have been shown to be temperature dependent[8,11,12], occurring freely above 15 °C, but being greatly restricted when temperatures are lowered below this point[2,11,13,14]. This has also been found to be a critical temperature range for the physical state of the lipid bilayer itself[15], with a phase change occurring from a fluid to a crystal-line gel as the temperature drops to about 15 °C. It is possible to influence the critical temperature at which this phase transition will occur. When this is done the temperature at which mobility of constituent macromolecules becomes restricted is comparably shifted[11]. Such alterations have been accomplished by influencing membrane fatty acid composition[11,19]; a higher degree of unsaturated fatty acids makes the achievement of a crystalline lipid state more difficult, so that membrane fluidity may be retained even at very low temperatures[16–18]. This has been done by growing cells in a serum supplement from which lipid has been removed, thereby shifting the temperature dependence of the mobility of receptor sites for concanavalin A on 3T3 and SV40-transformed 3T3 cells[11]. A similar technique was used in the present study.
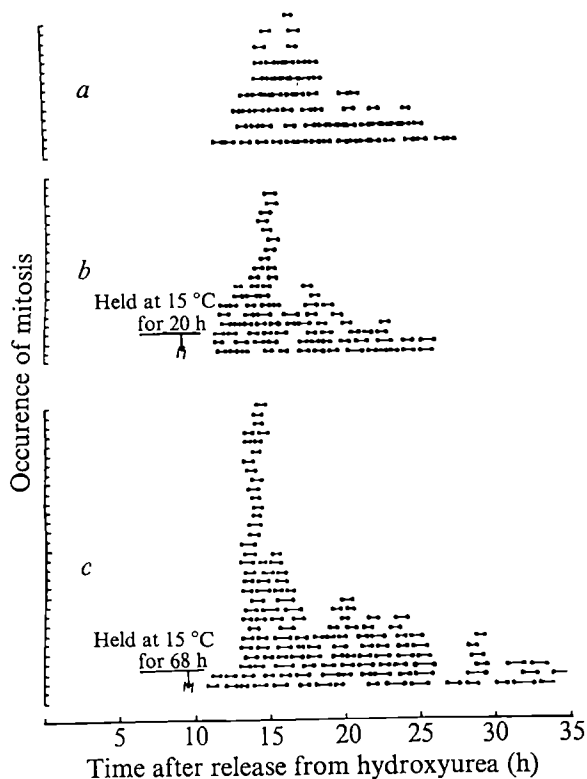
Fig. 1 Onset of mitosis after transferring cells from 15 to 37 °C. 3T6 cells were seeded at subconfluent densities (as cell–cell interactions might influence membrane transitions) in the absence of serum and incubated at 37 °C for 3 d. The G_1 arrested cultures thereby obtained were then transferred to rich serum conditions (10% foetal calf serum plus 5% calf serum), but in the presence of 2.88 mM hydroxyurea to hold the cells just at the border of entry into DNA synthesis. After incubation for 15 h in the presence of hydroxyurea, the cultures were washed and changed to fresh, serum-enriched, hydroxyurea-free medium. *a*, Filming was begun immediately after release from hydroxyurea. Incubation was at 37 °C throughout. *b*, Cultures were incubated at 37 °C for a further 9.5 h after hydroxyurea release and then transferred to 15 °C. After 20 h at 15 °C the cultures were shifted back to 37 °C and filming begun immediately *c*, As in *b* except cultures were left at 15 °C for 68 h. In the filming analysis, individual cells were followed and the times at which they rounded up (early metaphase) and divided into two attached daughter cells (late telophase) both recorded. Each horizontal bar starts at the rounded and extends through the double-cell stage for one cell. Each bar, therefore, represents the first mitotic event for one particular cell. The bars are placed on the graph in accordance with the time of occurrence of these events, and if the mitoses of two cells overlap in time, then one is placed above the other.

It has also been suggested that lectin receptors on transformed cells may generally be more amenable to mobilisation than are those of their untransformed counterparts[12,20] (see ref. 6 for review); although untransformed cells presumably take on a similar ease of surface rearrangement at about the time that they enter mitosis[21]. I have reasoned that if membrane reorganisation is a normal part of the progression of cells to mitosis, and if such reorganisation is contingent on an adequately fluid membrane environment, then interference with membrane fluidity should also prevent cells from entering mitosis. Since this sort of reorganisation may be expected to occur sometime between the completion of DNA synthesis and the onset of mitosis, then reversibly 'freezing' the membrane to prevent such movements from occurring, may be equivalent to reversibly arresting cells in the G_2 phase of growth.

I have attempted to 'freeze' movements within cell membranes by lowering the cultivation temperature of the mouse 3T6 cell line to 15 °C. Since a temperature decrease of this sort may be expected to have a variety of effects throughout the cell cycle[16,22–24], cells were first presynchronised to be as near as

possible to the completion of DNA synthesis before the temperature was lowered. Experimental conditions were thus intentionally manipulated with the specific purpose of looking at membrane-related events in G_2, rather than pursuing a general temperature effect on randomly growing cultures. Details of the presynchronisation are given in Fig. 1.

The mitotic responses of cells which had been held at 15 °C for various lengths of time and then shifted back to 37 °C are shown in Fig. 1. Here, mitoses were followed cinemicrographically, with the times of rounding up (early metaphase) and division into two attached cells (late telophase) recorded and plotted for each individual mitotic event. Figure 1*a* shows the profile for onset of mitosis after release of cells from hydroxyurea block and cultivation without interruption at 37 °C. The first mitoses occurred at about 12 h after release, with a broad peak between 14 and 19 h. If, at 9.5 h after release from hydroxyurea, cells were transferred to 15 °C for either 20 h (Fig. 1*b*) or 68 h (Fig. 1*c*) and then returned once again to 37 °C, the ensuing mitotic pattern was altered as shown. In both cases there was a lag of about 2 h before any mitoses were apparent at all. This time lag was not shortened by longer periods at the low temperature, and was followed by a very sharp mitotic burst occurring about 3–4 h after return to 37 °C. This burst was intensified by the longer period at the low temperature (Fig. 1*c*). It would seem from these observations that cells are being arrested, and not just retarded, in their movement through the G_2 phase, and that this arrest is reversible.

I attempted to confirm the conclusion that cells were being held in G_2 by low temperature by measuring the DNA content per cell on a BDH flow microfluorimeter. This is shown in Fig. 2 where the amount of DNA per cell in randomly growing cultures (S) is compared with that of cultures which had been presynchronised and then transferred to 15 °C (F10L63). There is a marked shift of the distribution in the F10L63 cells to the G_2 region. It can be roughly calculated from these distributions that in randomly growing cultures about 17% of the cells are in G_2, whereas in the F10L63 the G_2 population accounts for over 80% of the cells.

If the G_2 arrest is a function of the fluidity of some cellular membrane system, then the maintenance of high fluidity, even at low temperature, should enable the cells to proceed to mitosis even at 15 °C. As has been mentioned above, this can be attained by altering fatty acid compositions by cultivating the cells with a delipidated serum supplement. We treated foetal calf serum as detailed in Fig. 3 and then grew 3T6 cells in this

Fig. 2 DNA contents per cell of randomly-growing and cold-arrested 3T6 cells. Either randomly-growing cells (S) or cells which had been held at 15 °C for 63 h (F10L63) (as described for Fig. 1), were analysed for their DNA contents. Cells were fixed and stained with acriflavine according to the method of Tobet *et al.*[32] and the fluorescence per cell determined on a BDH flow microfluorimeter. The abcissa is in arbitrary fluorescence units, proportional to the amount of DNA, per cell and the ordinate represents the number of cells in that fluorescence channel. Between 1×10^6 and 2×10^6 cells were counted for both S and F10L63 cultivation conditions
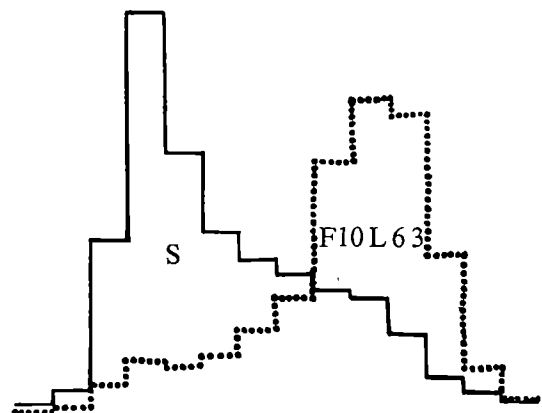
delipidated supplement, with an additional supply of biotin, (an essential cofactor for the biosynthesis of fatty acids) through several transfers at 37 °C. The fatty acids of cells which had been grown in normal serum and those which had been cultivated in the delipidated serum ('Delip' cells) were extracted[26] and analysed on a polyethylene glycol–adipate gas–liquid chromatography (GLC) column.

It was found that the proportion of saturated palmitate and stearate fatty acids in the 'delip' cells had decreased about threefold, whereas the unsaturated oleate plus elaidic fatty acids had proportionately increased about 2.5-fold compared with cells which had been grown in whole serum. To test whether this treatment also resulted in the release of cells from the low temperature G_2 block, the 'delip' cells were presynchronised and then shifted to 15 °C (Fig. 1). The DNA content/cell profiles for randomly growing 'delip' cells at 37 °C and cells held at 15 °C are shown in Fig. 3. Cells with the altered fatty acid composition were not held in G_2 at 15 °C. This was in agreement with the finding that the cell number of such cultures gradually increased at 15 °C, although no similar increase in number could be detected for cells which had been grown in whole serum.

I conclude that there is a temperature-dependent transition point in the G_2 phase of normal cell growth which is related to the fatty acid composition of some cell membrane system. I suggest that the G_2 function involved may be an obligatory surface rearrangement before mitosis, and that this rearrangement can be reversibly inhibited by decreasing membrane fluidity. Although fatty acid replacement experiments strongly implicate a fluid membrane function, one cannot identify definitively, from the present experiments, which of the cellular membrane systems may be involved[14,16]. Careful tailoring of membrane fatty acid compositions so as to adjust critical temperature transitions points to more physiological ranges should help both in elucidating this question as well as in providing a possible new method for sharply synchronising cells in the G_2 phase of growth.

I thank Dr Peter Riddle, Mr David Capallero and Miss Jyoti Gohil for assistance and Mr Michael Jones of the Chemistry Department of the ICR Fund for his help with the GLC fatty acid analyses.

MIKE SHODELL

Imperial Cancer Research Fund,
PO Box 123,
Lincoln's Inn Fields,
London WC2A 3PX, UK

1 Todaro, G. J., Lazar, G. K., and Green, H., *J. cell. comp. Physiol*, 66, 325–334 (1965).
2 Shodell, M., and Rubin, H., *In Vitro*, 6, 66–74 (1970)
3 Burk, R. R., *J. exp. Cell Res*, 63, 309–316 (1970).
4 Padilla, G. M., Cameron, I. L., and Zimmerman, A, *Cell Cycle Controls* (Academic, New York and London, 1974)
5 Tobey, R. A., *Nature*, 254, 245–247 (1975).
6 Nicolson, G. L., *Int. Rev. Cytol.*, 39, 90–190 (1974).
7 Edelman, G. M., Yahara, I, and Wang, J. L., *Proc. natn. Acad Sci. U.S A.*, 70, 1442–1446 (1973).
8 Edidin, M., in *Proc. ninth Can. Cancer Res. Conf* (edit by Scholefield, P G.), 95–113 (University of Toronto Press, 1972)
9 Singer, S J., *Adv. Immun.*, 19, 1–66 (1974)
10 Edidin, M., and Weiss, A., *Proc. natn Acad. Sci. U.S A.*, 69, 2456–2459 (1972).
11 Horwitz, A. F., Hatten, M E., and Burger, M M., *Proc natn Acad. Sci U.S A*, 71, 3115–3119 (1974).
12 Sachs, L., Inbar, M., and Shinitzky, M., in *Control of Proliferation in Animal Cells* (edit. by Clarkson, B, and Baserga, R.), 283–296 (Cold Spring Harbor Press, New York, 1974)
13 Rittenhouse, H. G, Williams, R. E., Wisnieski, B., and Fox, C F, *Biochem biophys Res. Commun.*, 58, 222–228 (1974).
14 Steim, J. M., *FEBS Symposia*, 28, 185–196 (1972)
15 Wisnieski, B. J., Parkes, J. G., Huang, Y. O, and Fox, C. F, *Proc natn. Acad. Sci. U.S A.*, 71, 4381–4385 (1974).
16 Lyons, J M, *Cryobiology*, 9, 341–350 (1972).
17 Phillips, M C., in *Progress in Surface and Membrane Science* (edit. by Danielli, J. F, and Rosenberg, M D.), 139–221 (Academic, New York and London, 1972).
18 Chapman, D., in *Biomembranes*, 4A, 123–158 (Plenum, New York, 1974)
19 Williams, R. E., Li, J K.-K., and Fox, C. F., *Biochem biophys. Res Commun*, 62, 462–469 (1975)
20 Raff, M. C., de Petris, S, and Mallucci, L., in *Control of Proliferation in Animal Cells* (edit. by Clarkson, B, and Baserga, R.), 271–281 (Cold Spring Harbor Press, New York, 1974)
21 Smets, L A, and Ley, L., *J. Cell Physiol.*, 84, 343–348 (1974)
22 Watanabe, I, and Okada, S., *J. Cell Biol.*, 32, 309–323 (1967)
23 Rao, P. N, and Engelberg, J., in *Cell Synchrony* (edit by Cameron, I. L., and Padilla, G. M.), 332–253 (Academic, New York and London, 1966)
24 Amos, H., Hoyt, R. F, and Horisberger, M, *In Vitro*, 6, 190–196 (1970).
25 Tobey, R. A., Crissman, H A., and Kraemer, P M, *J Cell Biol*, 54, 638–645 (1972)
26 Folch, J, Lees, M., and Sloan-Stanley, G H, *J. biol. Chem*, 226, 497–609 (1957).
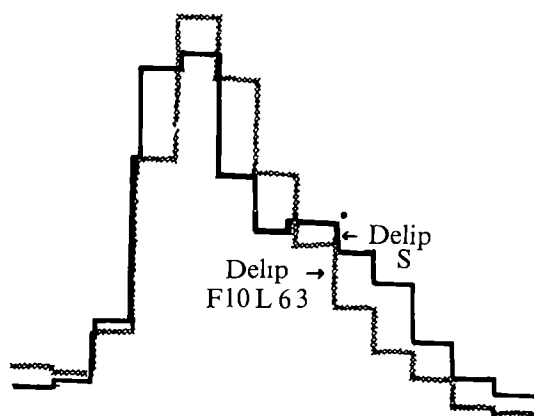
Fig. 3 DNA contents per cell of 3T6 cells with altered fatty acid composition. As for Fig. 2 except using cells which had been grown with a delipidated serum supplement. 100 ml of foetal calf serum were stirred with 100 ml acetone at 4 °C for 3 h. After centrifugation at 1,800g for 30 min, the cloudy yellow supernatant was discarded and the pellet resuspended in 150 ml chilled acetone and stirred in the cold overnight. This was then centrifuged, the supernatant discarded, and the sediment collected and dried under vacuum; this yielded 3.52 g of a white powder which could be stored for several months at −20 °C. For use in cell cultivation, 600 mg of the powder were suspended in 10 ml of 0.01 M acetate at pH 4.0 and then dialysed extensively against the acetate buffer in the cold. The suspension was then spun down at 1,800g for 2 h and the supernatant collected. (This was the delipidated serum and it contained ∼ 16.5 mg protein per ml.) The delipidated serum was filter-sterilised before use. S, Randomly growing cultures at 37 °C. F10L63, cells held at 15 °C for 63 hours.

Independent routes for Na transport across dog red cell membranes

PARKER and Snow[1] have shown that extracellular ATP produces an apparently nonspecific and reversible increase in the cation (Na^+ and K^+) permeability of dog red cells, as a result of which the cells swell gradually during suspension in an isosmolar physiological bathing medium containing ATP. This effect contrasts markedly with the usual dependence on cell volume of cation fluxes in this type of red cell[2–6] which, unlike most mammalian erythrocytes, is close to ionic equilibrium with plasma[6–7], and lacks an ouabain-sensitive ion pump[8,9]. Exogenous ATP was found to influence the surface properties of chick embryo fibroblasts[10], and also cause volume changes and cation imbalances in ascites tumour cells[11], and it has been suggested[11] that ATP alters the passive permeability of the membrane to Na^+ and K^+ rather than the cation pumping mechanism *per se*. Effects of ATP on the membrane transport characteristics of various tissue culture cell lines have been studied[12,13], and it has been proposed[12] that the translocation of ATP itself may be linked with ion movement across membranes.

I have now measured $^{24}Na^+$ uptake in dog red cells suspended in various media with and without extracellular ATP, and have found that the steady-state Na^+ influx, referred to cell volume in control isotonic conditions, is not changed significantly by swelling the cells in hypotonic media. In the presence of ATP, however, Na^+ permeability is greatly increased whether the cells are allowed to swell naturally or are shrunken by the osmotic effects of sucrose; in these circumstances it was necessary to estimate unidirectional Na^+ fluxes from the half times for $^{24}Na^+$ equilibration[6]. The sum of the Na^+ fluxes measured separately in cells suspended in isosmolar media

containing ATP, and in cells suspended in hyperosmolar media (+ 100 mM sucrose) in the absence of ATP, is equal to the Na^+ flux in ATP-treated cells after they have been shrunk. Shrinkage of the cells was carried out in the presence of ATP in media containing sucrose (100 mM). This indicates that the 'Na^+ channel' opened in the presence of ATP, when the cells are swollen, is not closed by shrinking the cells, and that the increase in the permeability to Na^+ normally brought about by shrinking the cells occurs by way of a route which is independent of and parallel to the pathway opened in the presence of extracellular ATP.

Quantitative measurements of $^{24}Na^+$ uptake, relative cell volume and intracellular Na^+ and K^+ concentrations were made from a series of single samples of packed cells spun down during the uptake of $^{24}Na^+$. ^{131}I-human serum albumin was used as the extracellular marker. Known volumes of packed cells were sampled with a Hamilton gas-tight syringe fitted with a Chaney adaptor. Details of the method have been described previously[5,6,14].

Figures 1 and 2 illustrate respectively the differences in the haemoglobin concentration (Hb_i) and the rate of uptake of $^{24}Na^+$ by cells in various experimental conditions. Cells suspended in media containing 25 mM and 50 mM less NaCl than the control isosmolar medium swelled by $13.1 \pm 0.7\%$ and $30.9 \pm 0.8\%$, respectively, and were at constant cell volume during the uptake of $^{24}Na^+$. In these conditions Na^+ influx was not altered significantly. By contrast, although the average volume of cells exposed to hyposmolar conditions (-50 mM NaCl) was similar to that of cells exposed to 2 mM ATP in an isosmolar medium (Fig. 1), in the latter group there was a 68-fold increase in Na^+ flux and a net uptake of Na^+ (Table 1).

When cells were suspended in a solution containing 100 mM sucrose, in addition to the usual constituents, they shrank by an average of $24.0 \pm 0.8\%$ and the Na^+ flux increased by a factor of 44 (Table 1). Although treatment with ATP had no significant effect on cell volume in the presence of 100 mM sucrose, the rate of Na^+ exchange was, however, increased even further to 395 ± 46 mEq per l cells (isosmolar cell volume) per h after ATP-treated cells had been shrunken. It is unlikely that the agreement between this value and the sum (437 ± 34 mEq per l cells per h) of the fluxes measured independently was fortuitous, as virtually the same findings were produced by separate experiments with red cells from two other dogs.

A secondary outcome of the present work was the lack of any discrepancy between the equilibrium levels of intracellular and extracellular specific activity of $^{24}Na^+$ in swollen cells treated with ATP. This indicated that the apparent sequestration of intracellular Na^+ in shrunken cells which was found in previous studies[5,6] was in fact the result of partial dehydration of the cells.

The effects of exogenous ATP and cell shrinkage on Na^+
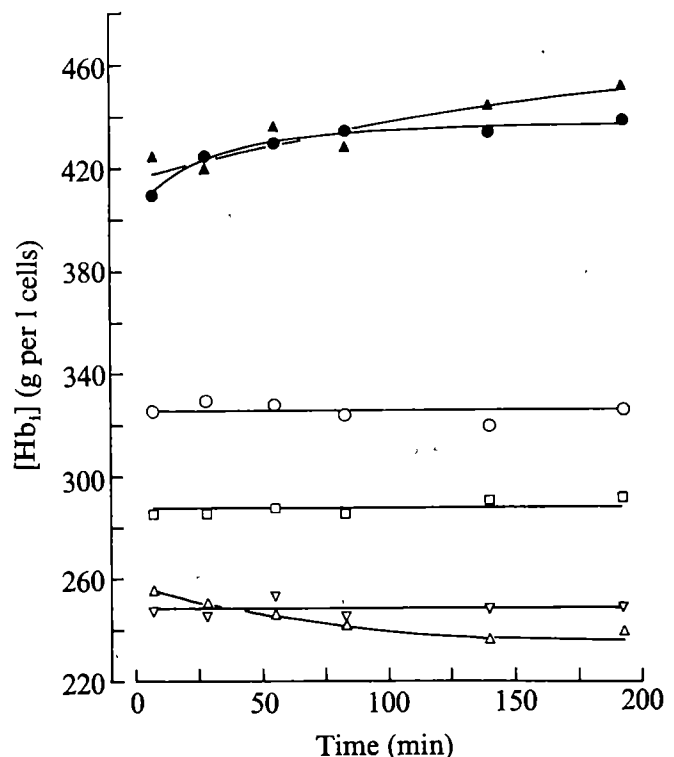
Fig. 1 Haemoglobin content of dog red cells during the uptake of $^{24}Na^+$ illustrated in Fig. 2. These data provide a measure of cell volume in the different media relative to that in control isosmolar conditions: NaCl, 137.2 mM; KOH, 5.0 mM; TES (N-*tris* [hydroxymethyl] methyl-2-aminoethane sulphonic acid), 5.0 mM; Na_2HPO_4, 5.2 mM; NaH_2PO_4, 0.8 mM; glucose, 5.0 mM; and 1% (w/v) bovine serum albumin (Armour, Cohn fraction V). Ca^{2+} and Mg^{2+} were omitted as they greatly inhibited the effect of ATP (ref. 1 and B. C. E., unpublished). pH of solutions containing ATP was adjusted to 7.2–7.4 with KOH (1.2 mM per mM ATP). Washed red cells were preincubated (where appropriate) in an isosmolar medium with 2.0 mM ATP for 2 h at 38 °C, then washed and resuspended in the same solution ($\pm$ sucrose 100 mM) at haematocrit values of about 10%. Control isosmolar, hyperosmolar and hyposmolar groups of cells were treated in a similar fashion with the appropriate bathing medium. Finally, all groups were gently shaken at 38 °C for 75 min in the presence of ^{131}I-albumin before the start of $^{24}Na^+$ uptake. ▲, +2 mM ATP, +100 mM sucrose; ●, +100 mM, sucrose; ○, control; □, −25 mM NaCl; ▽, −50 mM NaCl; △, +2 mM ATP.

fluxes are additive, implying that separate pathways are involved. There is, however, a common factor between the processes controlling the two states of increased permeability to Na^+, as it has been proposed[5] that phosphoglycerate kinase controls the dependence on cell volume of Na permeability in

---

Table 1 Na^+ fluxes in dog red cells as a function of cell volume in the presence and absence of ATP (2 mM)

Experimental conditions	Hb_i (g per l cells*)	Na^+_i (mEq per l cells*)	K^+_i (mEq per l cells*)	Normalised[‡] Na^+ flux (mEq per l cells per h)
Control	325.2 ± 1.4[†]	97.6 ± 0.3[†]	4.3 ± 0.1[†]	3.9 ± 0.1[§]
NaCl, −25 mM	287.6 ± 1.1	86.6 ± 0.4	3.9 ± 0.1	3.7 ± 0.1[§]
NaCl, −50 mM	248.5 ± 1.1	75.0 ± 0.1	3.4 ± 0.1	3.9 ± 0.1[§]
ATP, +2 mM	243.5 ± 4.0	107.0 ± 1.0	5.1 ± 0.1	265 ± 20[¶]
Sucrose, +100 mM	427.9 ± 4.1	108.2 ± 0.8	5.4 ± 0.2	172 ± 28[¶]
Sucrose, +100 mM ⎱ ATP, +2 mM ⎰	433.8 ± 5.0	99.6 ± 1.7	4.6 ± 0.1	395 ± 46[¶]

*Measured cell volume.

[†]Mean $\pm$ s.e. of six determinations in each column.

[‡]Fluxes referred to 1 l cells in control isosmolar conditions.

[§]Na^+ influx given by the ratio of the slope of the regression line fitted to the uptake of $^{24}Na^+$ (Fig. 2) and the extracellular specific activity of $^{24}Na^+$

[¶]Na^+ flux calculated from the rate constant of the exponential uptake of $^{24}Na^+$ (Fig. 2) using a two-compartment model[6]. s.e. of the fluxes were estimated from the s.e of the component parameters.
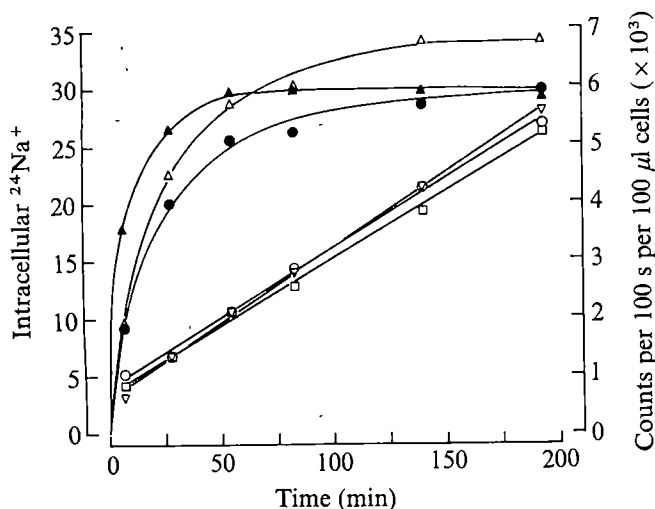
Fig. 2 Uptake of $^{24}Na^+$ by dog red cells in the conditions described in Fig. 1. Symbols as in Fig. 1. Right hand ordinate scale expanded fivefold for influx of $^{24}Na^+$ in control isosmolar conditions (O) and in hyposmolar media (−25 mM NaCl, □ and −50 mM NaCl, V). In groups ●, ▲, △ (all left-hand ordinate) and O, initial values of extracellular specific activity were almost the same (about 3.65×10^6 counts per 100 s per mEq Na^+); in groups □ and V the values were 4.23 and 5.32×10^6, respectively. Half times for the exchange of $^{24}Na^+$ were 7.6 ± 0.8 min (▲), 18.6 ± 3.0 min (●) and 20.4 ± 1.2 min (△), compared with about 780 min for groups V, O and □. Na^+ fluxes estimated from these plots are listed in Table 1.

[4] Romualdez, A , Sha'afi, R I., Lange, Y., and Solomon, A. K *J gen Physiol*, 60, 46 (1972).
[5] Elford, B C., and Solomon, A K , *Biochim biophys Acta*, 373, 253 (1974)
[6] Elford, B. C , *J. Physiol , Lond*, 246, 371 (1975)
[7] Berstein, R E , *Science*, 120, 459 (1954).
[8] Chan, P. C., Calabrese, V., and Theil, L. S , *Biochim biophys Acta*, 79, 424 (1964)
[9] Miles, P , R , and Lee, P., *J Cell Physiol* , 79, 367 (1972)
[10] Jones, B.M , *Nature*, 212, 362 (1966)
[11] Hempling, H. G , Stewart, C C . and Gasic, G , *J Cell Physiol.*, 73, 133 (1969).
[12] Trams, E' G., *Nature*, 252, 480 (1974)
[13] Alton, J F , and Lamb, J F., *J. Physiol , Lond* , 248, 14P (1975)
[14] Elford, B C., and Solomon, A K , *Nature*, 248, 522 (1974).
[15] Khan, M. M. T , and Martell, A E., *J Am. chem Soc.*, 88, 668 (1966)
[16] Caldwell, P C , in *Calcium and Cellular Function* (edit by Cuthbert, A W), (Macmillan, London, 1970)
[17] Davson, H , and Reiner, J. M., *J cell comp. Physiol* , 20, 325 (1942)
[18] Sha'afi, R L, and Pascoe, E , *J gen Physiol.*, 61, 709 (1973).
[19] Hille, B., *Progr. Biophys* , 21, 3 (1970).

the dog red cell; and this enzyme catalyses one of the ATP-producing reactions in the glycolytic sequence.

There is evidence[1] that exogenous ATP can alter the metabolic rate of dog red cells; however, it would seem that hydrolysis of the nucleotide is not providing a source of energy for the maintenance of the Na^+ channel, as ouabain (up to 10^{-3} M) has no inhibitory effect on the enhanced Na^+ flux in ATP-treated cells[1]. Note also that the ATP effect is probably not mediated by the removal of membrane-bound divalent cations, as up to 5×10^{-3} M EDTA, which chelates Ca^{2+} and Mg^{2+} more strongly than does $ATP^{15,16}$, has no effect *per se* on cell volume or Na^+ transport in dog red cells; neither does it interfere with the action of ATP in cells suspended in Ca^{2+}- and Mg^{2+}-free media (B. C. E., unpublished). Furthermore, it seems that ADP and other nucleotides are virtually without effect on cation permeability in the dog red cell[1].

The actual mechanisms of the volume-dependent and ATP-induced permeability changes are still unclear[1,4,5,11,12,17,18]; however, because of the large and to some extent cooperative increase in Na^+ flux caused by either ATP[1] or cell shrinkage[6] it is tempting to refer to Na^+ channels being switched on in the two circumstances. It would be extremely interesting if the properties of these channels were to bear any resemblance to the cation channels that have been characterised in nerve preparations[19]. Preliminary experiments, however, have indicated that tetrodotoxin (10^{-6} g ml^{-1}, pH 7.0) has no effect on the Na^+ channel opened in shrunken cells.

Obviously, cells are not going to encounter extracellular ATP concentrations of the order of 1–2 mM *in vivo*. It is clear, however, that ATP provides a specific way of perturbing membranes that gives rise to marked changes in permeability characteristics, not only of dog red cells but of a variety of cell types.

B. C. ELFORD

Division of Cryobiology,
Clinical Research Centre,
Harrow HA1 3UJ, UK

[1] Parker, J. C., and Snow, R. L., *Am. J Physiol* , 223, 888, (1972)
[2] Davson, H., *J. Physiol., Lond.*, 101, 265 (1942)
[3] Hoffman, J F., *Am. J. Med* , 41, 666 (1966)

State of molecular motion of cholesterol in lecithin bilayers

CHOLESTEROL is a major constituent of many biological membranes. Although its exact function is not completely understood, a large number of studies have indicated that cholesterol can affect the packing of lipid hydrocarbon chains, both in model and in biological membranes[1]. The interaction is envisaged to be such that above the thermal phase transition temperature (T_C), cholesterol inhibits flexing of the hydrocarbon chains, whereas below T_C it prevents the chains from crystallising into the rigid α gel[2-6].

Rothman and Engelman have argued[7] that the interactions between cholesterol and the phospholipid hydrocarbon chains in a lipid bilayer are primarily steric in nature, and the dramatic effects of cholesterol on the motional state of a phospholipid bilayer resides in the relative cross sectional area of the ring system (the nucleus) compared with that of the aliphatic tail. As the cross-sectional area of the nucleus is approximately twice that of the tail, steric interactions involving these parts of the molecule are thought to be different. If one supposes that in cholesterol-containing bilayer membranes, the upper part of the fatty acid chain is contiguous with the rather bulky cholesterol nucleus and the lower part of the chain with the narrower cholesterol tail, it seems plausible that cholesterol can serve to limit the number of accessible chain conformations along the upper part of the fatty acid chain while simultaneously increasing the number of accessible conformations to the lower part of the chain.

The question of cholesterol mobility is intriguing and important. Although the model of Rothman and Engelman[7], which views the cholesterol nucleus as being rather immobile and the tail as highly mobile, seems reasonable, it is primarily based on model building. Several nuclear magnetic resonance studies[6,8,9] disagree with this notion and claim that the cholesterol molecule as a whole, including the tail, is highly immobile, as a consequence of some specific 1:1 phospholipid–cholesterol complex formation. Unfortunately proton magnetic resonance spectra of cholesterol containing bilayer vesicles are not amenable to unambiguous interpretation. The spectrum is dominated by the broad methylene and methyl resonances of the phospholipid chains (Fig. 1a). Although cholesterol accounts for 25% of the total number of protons in a 2:1 lecithin–cholesterol mixture, none of its resonances could be discerned.

The molecular mobility of cholesterol in lipid bilayers is best ascertained by observing its proton magnetic resonance spectrum in conditions in which it is free of interference from the more intense hydrocarbon chain signals of the phospholipid molecules. The methylene and methyl resonances of the phospholipid chains can be suppressed if the fatty acid chains become totally deuterated. We have accordingly synthesised a series of $\sim 99\%$ deuterated lecithins for this purpose[10]. Figure 1b shows the corresponding spectrum of cholesterol in the deuterated L-α-dipalmitoylphosphatidylcholine (DPL) bilayer vesicles containing one part of cholesterol to two parts of lecithin at 75 °C. A cholesterol spectrum with rather well-defined features is obtained.

The most notable feature of the cholesterol spectrum is the
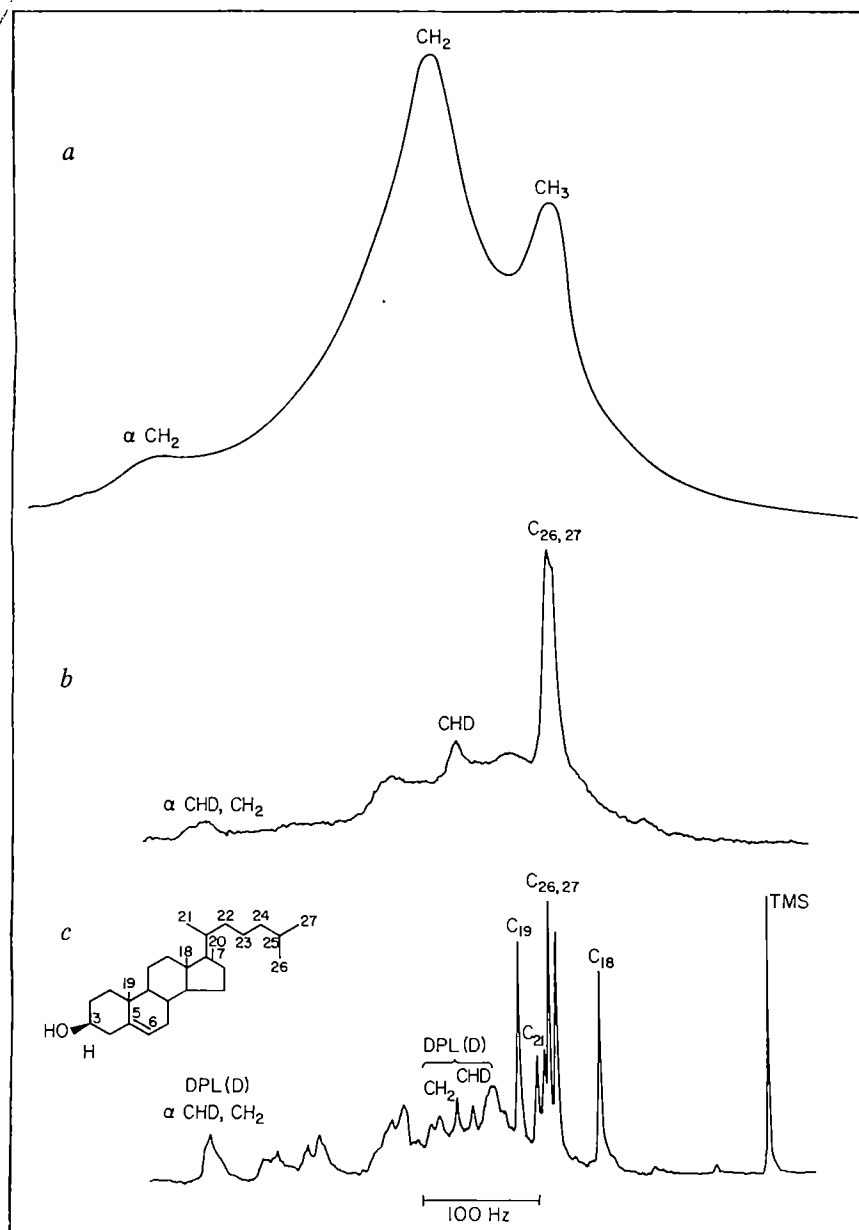
Fig. 1 Proton magnetic resonance (220 MHz) spectra of hydrocarbon chain protons of DPL vesicles containing cholesterol at 50 °C (a); cholesterol in deuterated DPL vesicles at 75 °C (b), sample b after freeze-drying and redissolved in deuterochloroform containing TMS at 20 °C (c). DPL–cholesterol molar ratio ~2:1 for all samples.

rather sharp, intense doublet which stands out amidst the broad background at −0.9 p.p.m. We assign this resonance by comparison with a spectrum of the same sample dissolved in $CDCl_3$ (Fig. 1c)[11]. There are five methyl groups in cholesterol and each of these shows up clearly in the chloroform spectrum (see spectral assignment indicated in Fig. 1c). Reference to this spectrum suggests that the sharp cholesterol resonance observed in the vesicle spectrum is to be assigned to the terminal C_{26}, C_{27} methyl groups. This assignment is confirmed by the field independent splitting, which is due to spin–spin coupling with the C_{25} methine proton. If this assignment is correct, essentially all the C_{26}, C_{27} methyl groups are contributing to this signal.

The remaining methyl resonances seem to be much broader, which indicates that their segmental motion is much more restricted than that of the isopropyl group of the tail. The degree of segmental mobility of cholesterol can be ascertained from the overall intensity of the cholesterol resonances appearing in the high resolution spectra, including the broader signals. Accurate intensity measurements of broad resonances in a high resolution spectrum are difficult, as the choice of baseline is always open to debate. Nevertheless, on a basis of the comparison of the total integrated intensity of the cholesterol resonances relative to that of the choline proton signal in our deuterated DPL, we conclude that no less than 70% of the cholesterol resonances including those of the C_{26}, C_{27} methyl

groups are observable at 75 °C. As the cholesterol sidechain protons correspond to only 50% of the total number of protons in the molecule, at least some of the cholesterol ring protons must be contributing to the spectrum. Thus cholesterol, when incorporated into a lipid bilayer, does not become totally immobilised. These results provide strong support for the Rothman–Engelman model.

The effect of temperature on the cholesterol spectrum is shown in Fig. 2. As the temperature is lowered the C_{26}, C_{27} methyl resonances show a gradual broadening, the linewidth increasing from ~13 Hz at 83.4 °C to ~23 Hz at 54.5 °C. These observations, we believe, reflect the expected gradual increase in the segmental order and/or a decrease in mobility of the tail as the temperature is decreased. It is significant, however, that even at 26°C a cholesterol spectrum could still be discerned, although the resonances were now very broad. There is thus significant residual segmental motion of the cholesterol molecule even at temperatures 11 °C below the thermal phase transition temperature of DPL[10]. In contrast, small DPL vesicles revealed no high resolution hydrocarbon chain resonances at this temperature in the absence of cholesterol.

Engelman and Rothman[4] have also proposed that a phase boundary occurs in DPL–cholesterol mixtures at 20 °C in the region near 33 mol % cholesterol. To ascertain the existence of this or a similar phase boundary at higher temperatures, we have examined mixed cholesterol–lecithin systems at two
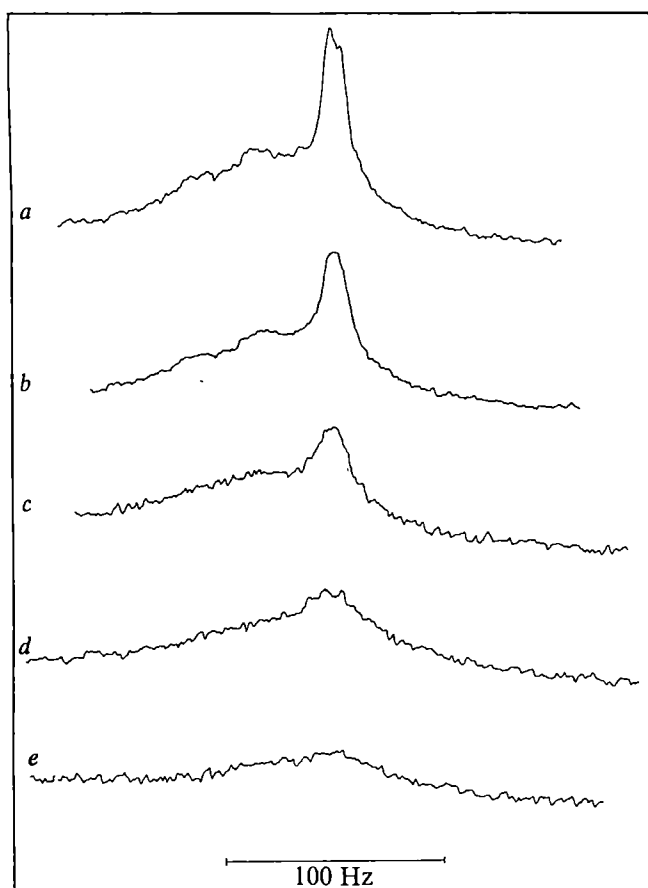
Fig. 2 Effect of temperature on 100 MHz proton magnetic resonance spectra of cholesterol in deuterated DPL vesicles. DPL-cholesterol molar ratio ~2:1. *a*, 83.4; *b*, 69.5; *c*, 54.5; *d*, 36.5; *e*, 26 °C.

2 Demel, R. A., van Deenen, L. L. M., and Pethica, B A , *Biochim. biophys. Acta*, 135, 11–19 (1967).
3 Hinz, H. J., and Sturtevant, J M., *J biol. Chem* , 247, 3697–3700 (1972).
4 Engelman, D. M., and Rothman, J. E., *J. biol. Chem* , 247, 3694–3697 (1972).
5 Shimshick, E. J , and McConnell, H. M , *Biochem biophys. Res. Commun.*, 53, 446–451 (1973).
6 Phillips, M. C., and Finer, E. G , *Biochim. biophys. Acta*, 356, 199–206 (1974)
7 Rothman, J. E., and Engelman, D. M., *Nature new Biol* , 237, 42–44 (1972)
8 Darke, A., Finer, E. G., Flook, A. G., and Phillips, M. C., *FEBS Lett.*, 18, 326–330 (1971)
9 Darke, A., Finer, E G , Flook, A. G., and Phillips, M C., *J. molec Biol* , 63, 265–279 (1972).
10 Petersen, N. O , Kroon, P. A., Kainosho, M , and Chan, S I., *Chem Phys Lipids* (in the press).
11 Slomp, G , and MacKellar, F. A., *J. Am chem. Soc.*, 84, 204–206 (1962)

concentrations corresponding to DPL-cholesterol molar ratios of 2:1 and 1:1. Proton magnetic resonance measurements of these two bilayer systems at 83.4 °C (and at other temperatures) revealed no significant difference between the spectra for the two samples, except for an intensity deficiency in the cholesterol C_{26}, C_{27} methyl peaks for the sample more concentrated stoichiometrically in cholesterol. Whereas intensity measurements of the sharp cholesterol methyl signals relative to that of the choline methyl peak of the lecithin indicated that all the cholesterol methyl signal intensity expected is being observed for the 2:1 mixture, 30% of the cholesterol methyl intensity is not accounted for in the 1:1 mixture. This result suggests the existence of a phase boundary in lecithin-cholesterol bilayers at a cholesterol concentration of ~40%.

In summary, we have obtained experimental evidence for a differential mobility of the cholesterol nucleus and its aliphatic tail when cholesterol is incorporated into lecithin bilayer vesicles. The motion of the isopropyl moiety of the tail was shown to be essentially unrestricted, and exhibited a high degree of mobility even at temperatures well below the thermal phase transition of the phospholipids into which it is incorporated. We have also obtained evidence for the existence of a phase boundary in mixed cholesterol-lecithin systems in the region near 40 mol % cholesterol.

This work was supported by the National Institute of General Medical Sciences, US Public Health Service.

Paulus A. Kroon
Masatsune Kainosho
Sunney I. Chan

A. A. Noyes Laboratory of Chemical Physics,
California Institute of Technology,
Pasadena, California 91125

1 Oldfield, E., and Chapman, D., *FEBS Lett.*, 23, 285–297 (1972).

Effects of structural defects in sonicated phospholipid vesicles on fusion and ion permeability

ULTRASONICATED lecithin-water dispersions, consisting of single-walled bilayer vesicles, are used as a model for the phospholipid bilayer in biomembranes and have the unique advantage of a well-defined inside and outside[1,2]. The system is well suited, therefore, for studies of transport of solutes across the bilayer membrane[3]. Fusion of these structures may also be used as a model for cell fusion, endocytosis and related phenomena[4,5].

Small sonicated vesicles, because of their large surface curvature, are known to exhibit different physical properties from large bilayer vesicles and multilamellar liposomes[2]. In particular, there is a tendency for the size distribution of a vesicle suspension to change spontaneously by the process of vesicle fusion in certain temperature conditions[6]. As the definition and control of this model membrane system are important, factors influencing the stability of bilayer vesicles are of interest. Here we describe an annealing phenomenon which we have found to be necessary for the stability of vesicle suspensions.

Variations in the vesicle size distribution can be monitored by changes in the proton magnetic resonance spectrum. Previous studies have demonstrated that for large vesicles the proton magnetic resonance spectrum of the hydrocarbon chain protons is broadened compared with that for small sonicated vesicles, together with a concomitant loss of intensity[2]. In addition, small sonicated vesicles exhibit distinct inner and outer choline methyl signals at high magnetic fields because of different surface curvatures between the two halves of the bilayer membrane[7]. This chemical shift non-equivalence enables perturbations on the inner and outer halves of the bilayer to be monitored separately. Large single-walled vesicles which do not possess this structural difference between the two halves of the membrane, however, do not exhibit this chemical shift non-equivalence, but the addition of a paramagnetic shift reagent to the external solution after preparation results in well separated choline signals provided the bilayer membrane is impermeable to the paramagnetic ion[8].

Small sonicated phospholipid bilayer vesicles are usually obtained by prolonged sonication of a phospholipid dispersion at high sound intensity. In view of the high power input to the sample, the local temperature of the solution frequently reaches well above the thermal phase transition (T_C) of the lipids. Recently we have found that vesicles with a controlled range of average sizes can be prepared directly by mild sonication at low temperatures $(< T_C)$, but that subsequent heating of the resultant sonicated lecithin dispersion to a temperature above T_C is an absolute prerequisite for obtaining a bilayer structure which is impermeable to ions. Moreover, there was a pronounced tendency for the sonicated vesicles to fuse, with the rate of fusion depending strongly on whether they have been annealed or not. These observations, we feel, have bearing on a number of reports regarding the rapid fusion of small sonicated bilayer vesicles[6,9].

Figure 1 illustrates both the effects of annealing on the permeability of bilayer vesicles to Eu^{3+} ions and on the rate of vesicle fusion. When $Eu(NO_3)_3$ was added in isotonic conditions
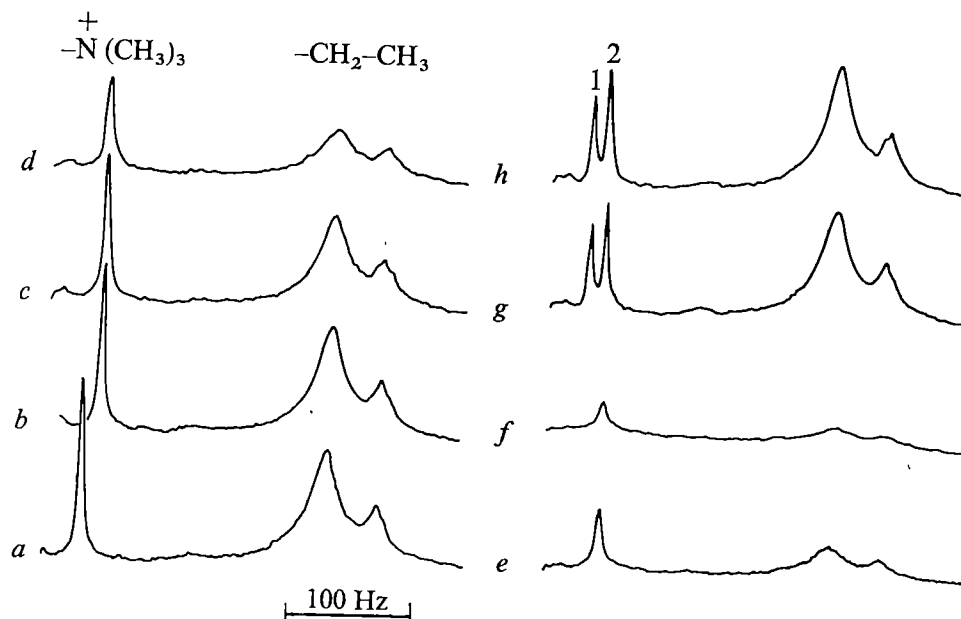
Fig. 1 DSL (2 5 w/v%), purified by a silicic acid column chromatography, was sonicated at high power in a 5 mM solution of $La(NO_3)_3$ in D_2O using a Branson sonicator with Ti microtip. To maintain the solution below T_C during (~56 °C) sonication, it was cooled externally by an ice-water bath, and an alternating sonication sequence of 30-s periods of sonication and 30-s intervals was used After a total of 15 min a practically transparent solution was obtained. This was divided into eight 5-mm nuclear magnetic resonance sample tubes, which were kept at different annealing temperatures (see below) for 30 min The samples were then brought to room temperature (23 °C) and equal volumes of 5 mM $Eu(NO_3)_3$ solutions were added immediately to all the samples, except for the control sample (*a*) to which a 5 mM $La(NO_3)_3$ solution was added. After thorough mixing by shaking, all samples were kept at 69 °C, the nuclear magnetic resonance probe temperature, until the nuclear magnetic resonance spectrum was recorded. The latter step was introduced to fix the state of all the samples before recording spectra. Fourier transform ^1H nuclear magnetic resonance spectra were obtained with a Varian XL-100 spectrometer The temperatures (°C) at which the samples were kept before addition of the La salt solution (the annealing temperature) were: *a*, 23; *b*, 23; *c*, 42; *d*, 49.5; *e*, 52.4, *f*, 55 6 *g*, 59 2; *h*, 63. The spectral assignments indicated correspond to the choline methyl protons ($-N^+(CH_3)_3$) and the methylene and methyl proton of the hydrocarbon chain (CH_2, CH_3). 1 and 2, Choline methyl signals associated with the inside and outside halves of the bilayer respectively. Measurements of the signal intensities of the choline methyl resonances for samples *a*, *b*, and *h* indicate that in excess of 90% of the expected intensities are being observed.

to samples of L-α-distearoylphosphatidylcholine (DSL) or L-α-dipalmitoylphosphatidylcholine (DPL) at room temperature after sonication, the proton magnetic resonance spectrum of these vesicles above T_C exhibited only one methyl signal for both the inner and outer choline groups. The spectral position of this choline signal was shifted to higher fields relative to that in a similar preparation containing only $La(NO_3)_3$ in both the intra and extravesicular solutions, indicating leakage of Eu^{3+} ions into the internal compartment of these vesicles in these conditions. In contrast, when another portion of the same sonicated preparation was heated above T_C before Eu^{3+} ions were added to the solution at room temperature, the proton magnetic resonance spectrum of these vesicles revealed two choline methyl peaks. A more detailed study of this phenomenon involving preheating samples at various temperatures (Fig. 1) after sonication and cooling of the sample back to room temperature before the addition of the shift reagent has led us to conclude that annealing of the vesicles above the thermal phase transition is absolutely essential to obtain bilayers which are impermeable to ions.

Bilayer vesicles which have not previously been annealed above T_C have been found to undergo extensive fusion. The fusion rate increases with increasing temperature and seems to reach a maximum at the thermal phase transition temperature. This vesicle fusion is manifested in the proton magetic resonance spectra in a pronounced way. There is a conspicuous absence of intensity in the high resolution spectra. As the observed spectra do not seem to be significantly broadened from those for small sonicated bilayer vesicles, this intensity anomaly may be caused by polydispersity of the final vesicle suspension as a result of different extents of annealing in the original preparation.

Our experiments also revealed a correlation between the permeability of these bilayers to ions and their tendency towards fusion. As annealing starts around T_C, the bilayer membrane becomes less permeable to Eu^{3+} ions. The fusion rate also goes down abruptly. Because of the relationship between annealing and fusion, kinetic experiments on annealing are somewhat difficult to interpret. Nevertheless it seems that annealing is complete within 2 h if it is carried out at 3 °C above T_C. At higher temperatures the annealing is even faster; for example, annealing is completed within 10 min at 10 °C above T_C After the vesicles are annealed, fusion was extremely slow. For annealed samples of DPL vesicles, proton magnetic resonance and turbidity measurements revealed no evidence for fusion, even when they were kept for 16 h at 42 °C (T_C). Thus the fusion of phospholipid bilayer vesicles depends strongly on the history of the sample with respect to annealing.

The above observations can be accounted for by structural defects within the bilayer structure after sonication below T_C. The high permeability of unannealed structures towards ions may be caused by imperfect boundaries between otherwise homogeneous lipid domains. In fact, we have evidence for structural differences between annealed and unannealed bilayer samples below T_C. Figure 2 shows proton magnetic resonance spectra of a DSL sample before and after annealing (15 min at 67 °C) and recorded at 50 °C (below T_C). Between the unannealed and the annealed sample we observed ~50% increase in the intensities together with a pronounced spectral sharpening of the hydrocarbon chain signal. Identical observations were obtained for DPL. These observations suggest that the unannealed bilayer vesicle below T_C consists of a discontinuous arrangement of highly ordered lipid domains. Unannealed bilayer vesicles could also be permeable to Eu^{3+} because of a highly fluid, but homogeneous bilayer structure. This possibility seems unlikely since such a bilayer membrane would be expected to yield sharper resonances before, rather than after, annealing—just the reverse of what is observed (Fig. 2). Electron microscopic studies also support the idea of cracks in the bilayer structure between well ordered lipid domains. It has not been possible so far to obtain electron micrographs for unannealed phospholipid vesicles by the negative staining
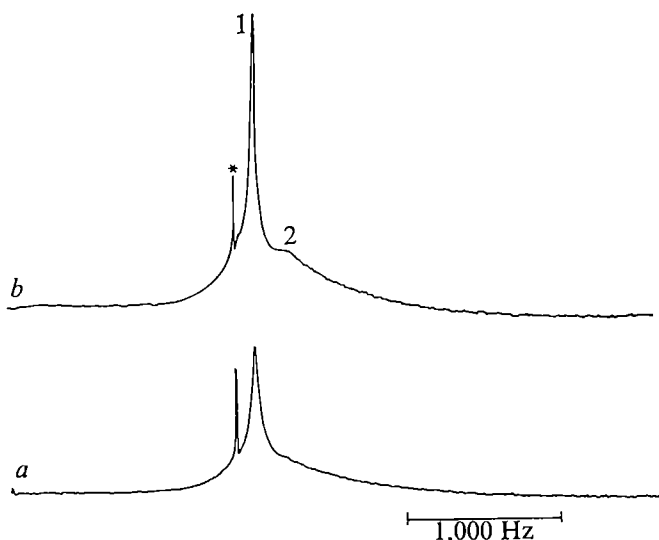
Fig. 2 Fourier transform ^{1}H nuclear magnetic resonance spectra (100 MHz) of the sonicated DSL solutions (2.5 w/v%) in D_2O containing 5 mM $La(NO_3)_3$. Procedure of sonication as described in Fig. 1. Sample b was the same as a except that it has been annealed for 15 min at 67 °C. Nuclear magnetic resonance measurements were made at 50 °C, which is below the phase transition temperature of DSL ($\sim$56 °C), to prevent further annealing during the nuclear magnetic resonance measurement. 1 and 2, Choline methyl and aliphatic methyl and/or methylene signals respectively. *Residual HDO signal, which was partially saturated by the fast pulsing cycle.

method, presumably because the staining solution leaks through these regions in the bilayer structure.

The above observations regarding the relationship between annealing and fusion were observed for DSL, DPL and L-α-dimyristoylphosphatidylcholine (DML). Similar results were obtained irrespective of whether the studies were carried out in pure D_2O or in NaCl solutions, although the vesicle dispersions in these cases were not as clear as in the presence of La^{3+} ions, suggesting that electrostatic surface conditions may influence the formation of vesicles during the sonication procedure. There is a pronounced difference between purified (by silicic acid column chromatography) and unpurified samples in that the fusion rate is much higher for the purified lipids. Rapid fusion of DML and DPL vesicles at the phase transition temperature of these lipids has been reported[6,9]. The problem of annealing, however, especially its effect on fusion, was unknown.

Measurements on mixed lipid systems show analogous results. For a mixture of DPL and DSL (1:1 by weight) the appropriate annealing temperature and the temperature associated with the maximum fusion rate was found to be about 49 °C, midway between T_C for. the respective pure lipids, suggesting ideal mixing within the lipid domains. Other mixed lipid systems, such as DPL with L-α-dipalmitoylphosphatidylethanolamine (DPE), cholesterol or sphingomyelin (bovine brain), show similar behaviour.

In support of our proposal of rapid fusion of unannealed bilayer vesicles, comparative absorbance measurements on mixtures of DSL and DPL vesicles indicated that such mixtures of lipid vesicles exhibit distinct differences in their behaviour on heating, depending on whether the vesicles were annealed or not before mixing of the otherwise homogeneous suspensions. Only when both the DSL and DPL vesicle suspensions were mixed in the unannealed state have we obtained evidence for cross fusion between DSL and DPL vesicles.

Our results, therefore, may have implications for proposed effects of lateral phase separation on the permeability of a bilayer membrane[10-12]. In particular an enhanced Na^+ permeation through membrane vesicles at the lipid phase transition temperature has been reported, and it was proposed that boundary regions between the solid and liquid crystalline phases facilitate this permeability increase[11]. Whether these

boundaries between coexisting solid and liquid crystalline phases at T_C are similar to the metastable structural defects below T_C proposed here is now under investigation.

RUDIGER LAWACZECK
MASATSUNE KAINOSHO
JEAN-LUC GIRARDET*
SUNNEY I. CHAN

*A. A. Noyes Laboratory of Chemical Physics,
California Institute of Technology,
Pasadena, California 91125*

1 Huang, C., *Biochemistry*, **8**, 344–351 (1969).
2 Sheetz, M. P., and Chan, S. I., *Biochemistry*, **11**, 4573–4581 (1972).
3 Papahadjopoulos, D., Nir, S., and Ohki, S., *Biochim biophys. Acta*, **266**, 561–583 (1972).
4 Papahadjopoulos, D., Mayhew, E., Poste, G., Smith, S., and Vail, W. J., *Nature*, **252**, 163–165 (1974).
5 Maeda, T., and Ohnishi, S., *Biochem. biophys. Res. Commun*, **60**, 1509–1516 (1974).
6 Prestegard, J. H., and Fellmeth, B., *Biochemistry*, **13**, 1122–1126 (1974).
7 Kostelnik, R. J., and Castellano, S. M., *J. magn Resonance*, **9**, 291–295 (1973).
8 Bystrov, V. F., Dubrovina, N. I., Barsukov, L. I., and Bergelson, L. D., *Chem Phys. Lipids*, **6**, 343–350 (1971).
9 Taupir, C., and McConnell, H. M., *Mitochondria/Biomembranes*, Ninth FEBS Symp., **28**, 219–229 (1972).
10 Shimshick, E. J., and McConnell, H. M., *Biochemistry*, **12**, 2351–2360 (1973).
11 Papahadjopoulos, D., Jacobson, K., Nir, S., and Isac, T., *Biochim biophys Acta*, **311**, 330–348 (1973).
12 Haest, C. W. M., DeGier, J., Van Es, G. A., Verkleij, A. J., and Van Deenen, L. L. M., *Biochim biophys Acta*, **288**, 43–53 (1972)

Neurofilament disguise, destruction and discipline

NEUROFILAMENTS $\sim$100 Å in diameter appear in electron micrographs to be major constituents of most nerve cells, but their function is not clear and their shapes vary[1]. In thin sections of brain they appear relatively thick and knobby[1]; whereas in negative stain, squid neurofilaments appear smooth and cylindrical[2,3]. Only neurofilaments from the squid giant axon[3] and from calf brain[4,5] have been isolated in quantities sufficient for analysis, but these differ in their solubility properties and subunit molecular weights. These inharmonius observations are partially clarified by the transformations reported here of neurofilaments from a new and nearly ideal source, the giant axon of a marine worm, *Myxicola infundibulum*.

Myxicola axoplasm can be extracted from the worm in about 10 s (ref 6) (compare squid, 20 min; calf, 3 h), an important factor in view of the time-dependent changes reported here. *Myxicola* axoplasm is a highly structured gel consisting almost exclusively of neurofilaments[6,7,17]. We have detected no microtubules. Electron microscopy (Fig. 1) indicates that *Myxicola* neurofilaments assume a variety of shapes when processed in different ways. Reasonable care has been taken to identify and control the pitfalls of electron microscope assay, such as sampling errors, and staining, drying and proteolytic artefacts

In thin sections, *Myxicola* neurofilaments resemble most other neurofilaments[1], if fixed in the usual manner; that is, with glutaraldehyde, while inside an intact axon (Fig. 1a; diameters 70–280Å). Finer fibrous arrays (Fig 1b; diameters 15–120 Å), resembling microfilaments[1], appear throughout the axoplasm, if it is fixed after extraction from the axon. Though the fixatives used were identical, the environments of the proteins during fixation and the rates of fixation probably differ in Fig. 1a and b, because the membranes surrounding the axon act as a considerable diffusion barrier in Fig. 1a, but are absent in Fig. 1b. Figure 1, a and b
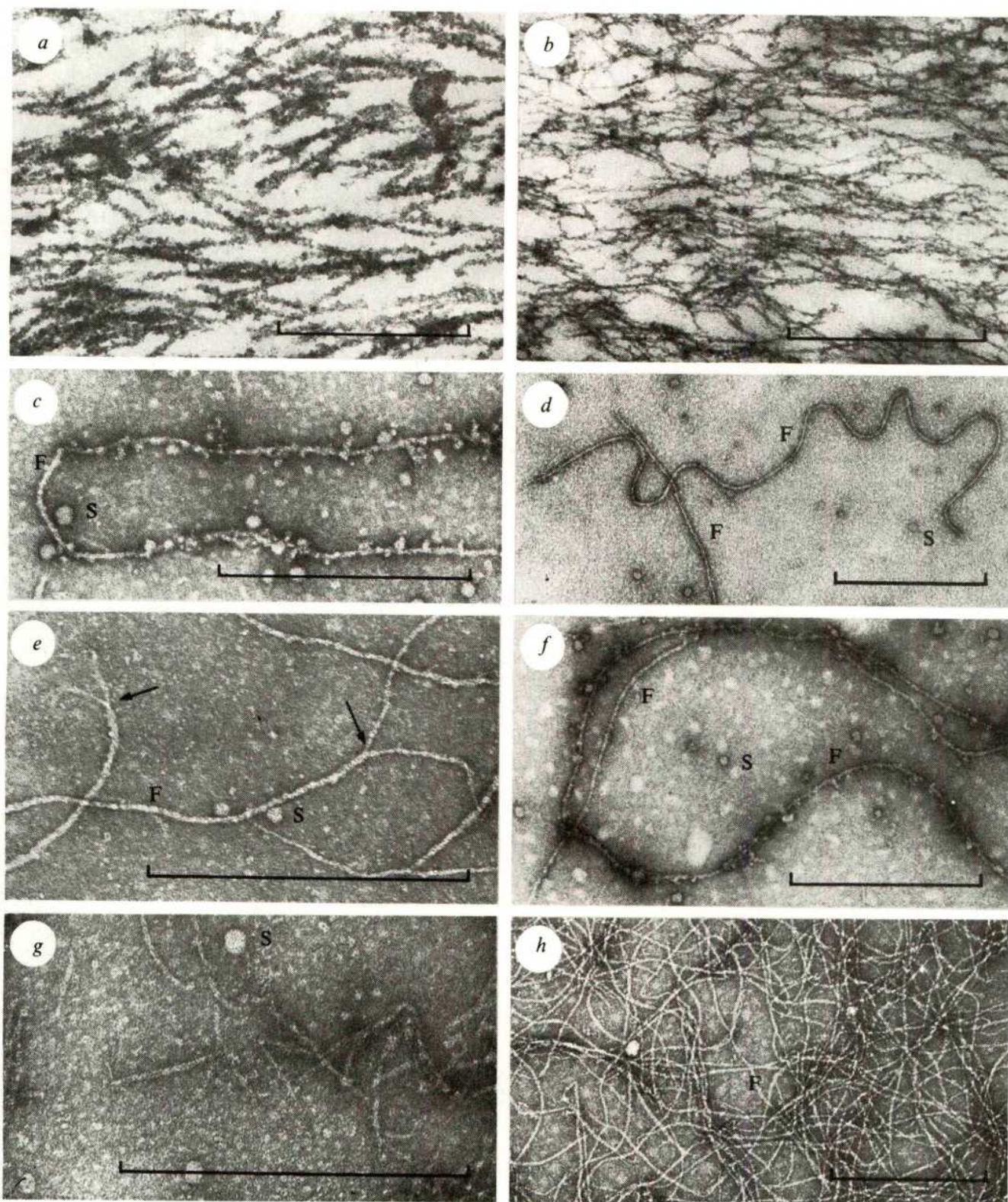
Fig. 1 Electron micrographs of *Myxicola* neurofilaments. *a*, *b*, Thin sections; *c–h*, negatively stained with 1% uranyl acetate on carbon-formar coated grids. *a*, Whole ventral cord fixed for 90 min (2% glutaraldehyde, 70 mM cacodylate, *p*H 7.2, adjusted to 1.01 osmolal with triple-strength artificial seawater), 23 °C. Postfixed with 1% OsO₄ with similarly adjusted tonicity and *p*H. Dehydrated in ethanol, embedded in Araldite, sectioned, and stained with uranyl acetate and lead citrate. Examined on a Siemens 1A at 80 kV. *b*, Extracted axoplasm, processed as in (*a*). *c*, Extracted axoplasm rinsed in buffer (20 mM cacodylate, *p*H 7.0) fixed 30 min in the above fixative, and homogenised in buffer. *d–f*, Extracted axoplasm homogenised (*d*) in buffer, (*e*) in distilled water, showing frayed filaments (→), and (*f*) in buffer containing 5% sucrose. *g*, Extracted axoplasm homogenised in buffer containing 0.5 M KCl; then rapidly diluted with 5 volumes of water, and applied to the grid. *h*, Reconstituted filaments, from the same fraction described in Fig. 2*f*. Calibration bar 0.5 μm. Abbreviations: F, ∼ 70 Å diameter filament; S, ∼ 280 Å diameter sphere.

indicates that the state of aggregation of the neurofilament proteins can be altered. Negatively-stained preparations (Fig. 1, *c–h*) point to the same conclusion.

Fixation (as for either Fig. 1*a* or *b*), followed by homogenisation and negative staining, yields filaments (F) with irregular, globular projections, as well as roughly spherical particles (S), which often appear attached to the filaments (Fig. 1*c*). If fixation is omitted, or occurs after homogenisation, most filaments have no projections, and detached spheres are numerous (Fig. 1*d*). Filaments are 72 ± 10 Å diameter (mean $\pm$ s.d.), and up to 10 μm long. Spheres are 280 ± 40 Å diameter. Both are proteins, since they are rapidly destroyed by Pronase. Filaments usually taper at their ends, but sometimes appear to untwist and fray into finer fibrous components ($\sim$15–50 Å in diameter, Fig. 1*e*, arrows). Sucrose and glycerol solutions often show projections and spheres attached to the filaments (Fig. 1*f*). High ionic strength solutions (μm$>$0.5) reversibly dissociate both worm (Fig. 1*g, h*) and squid filaments[2,8], but not those from brain[4,5].

Of the several possible interpretations of the above data, we favour this: 70 Å filaments consist of a twisted array of fibrous, largely α-helical[7] subunits, linked primarily by electrostatic bonds, since they are dissociated in solutions of high ionic strength. Non-covalently bonded to the 70 Å filaments are long branches (not easily distinguished from the filaments in Fig. 1*b*), which can coalesce with either the filament, to form the small projections seen in Fig. 1*a, c* and *f*, or with each other, to form the larger projections, and the attached and detached spheres seen in Fig. 1*c–h*.

Sodium dodecyl sulphate (SDS) gel electrophoresis of whole *Myxicola* axoplasm gives a different, and less complex, pattern of polypeptide chains than whole squid (*Loligo*

forbesi) axoplasm (Fig. 2*a, b*). Whole *Myxicola* axoplasm has two dominant bands on SDS gels (Fig. 2*b*) corresponding to chain weights of $152,000 \pm 5,000$ (mean $\pm$ s.d., $n=19$; roughly 40% of total protein), and $160,000 \pm 4,000$ (roughly 10% of total protein). (Standards used for molecular weight determinations were: myosin, c-protein, cross-linked actin, tubulin, bovine serum albumin, lactate dehydrogenase, β-galactosidase and phosphorylase a.) Neither band comigrates with a dominant band from squid axoplasm.

Three observations suggest that *Myxicola* neurofilaments are composed of both the 152,000 and the 160,000 chains, together with lesser amounts of other chains, as shown in Fig. 2*c, d* and *f*. First, and most generally, electron microscopy indicates that neurofilaments account for the great majority of proteins in axoplasm, and these bands account for most of the protein on SDS gels. Second, and more specifically, repeatedly washed *Myxicola* neurofilaments (Fig. 2*d*) show bands similar to those of whole axoplasm, and in approximately their original proportions. Some heterogeneous and largely low molecular weight material is removed by this procedure (Fig. 2*e*). Third, neurofilaments reconstituted after dissociation in high salt concentrations show the same set of bands (Fig. 2*f*). We have not yet eliminated the possibility of contaminants either copurifying, or adsorbed to the neurofilament subunits; nor have we yet identified particular bands with each of the structural components of the neurofilament.

Calcium ions cause major changes in *Myxicola* axoplasm. Incubation with calcium for as little as 15 s causes a shift in the neurofilament gel pattern to lower molecular weight positions (Fig. 2*g–i*). Negatively-stained Ca²⁺-treated filaments fray into fine fibres, then decompose further into ill-defined, flocculent clots. We interpret these events as the action of a Ca²⁺-activated protease, present in whole axoplasm. Washing axoplasm (as for Fig. 2*d*) removes this protease, since washed filaments with added 10 mM Ca²⁺ are stable (as judged by SDS gels and electron microscopy), but alter as described above if the first wash supernatant (Fig. 2*e*) is then added. The protease can be inhibited by PCMB (parachloromercuribenzoate, 1 mM), or TPCK (L-1-tosylamide-2-phenylethylchloromethyl ketone, 1 mM), or TLCK (N-α-*p*-tosyl-L-lysine chloromethyl ketone HCl, 1 mM), but not by PMSF (phenylmethylsulphonylfluoride, 1 mM). No digestion has been detected in the absence of added Ca²⁺, for incubations up to 90 h.

Calcium ions (1–10 mM) also cause a macroscopic, rapid dispersal of whole *Myxicola* and squid[9] axoplasm in water. PCMB (1 mM) inhibits this effect in *Myxicola*, so this seems to be caused by the Ca²⁺-activated protease. Using SDS gels and electron microscopy, we have confirmed the presence of a similar PCMB- and EDTA-inhibited protease in squid

Squid   *Myxicola*

Origin

160,000
152,000

65,000

a b c d e f g h i

Light load
Heavy load
Washed
Supernatant
Reconstituted
1 mM Ca²⁺
5 mM Ca²⁺
20 mM Ca²⁺

Whole axoplasm

Fig. 2 SDS gel electrophoresis on 5% polyacrylamide of neurofilament preparations, essentially according to Weber and Osborne[17]. *a*, Whole squid axoplasm; *b, c*, whole worm axoplasm; *c*, heavier loading of worm axoplasm, showing minor components. *d*, Washed neurofilaments, prepared by homogenising axoplasm in buffer (20 mM histidine, 0.1 mM PMSF, *p*H 7.0), centrifuging 2 h at 150.000*g*, resuspending the pellet, and repeating this cycle four more times. A low speed spin (20,000*g*; 15 min) was also included to remove particulate matter. The final pellet was used for (*d*), and the first supernatant is shown in (*e*). *f*, Reconstituted neurofilaments, prepared by homogenising axoplasm in buffer containing 0.75 M KCl, removing particulate matter (20,000*g*; 15 min), centrifuging (1.5 h; 250,000*g*), then dialysing the supernatant against buffer containing 0.1 M KCl, and sedimenting the reconstituted neurofilaments (2 h; 150,000*g*). *g, h* and *i*, Whole axoplasm after incubation (40 min, 20 °C) with calcium ions. 20 mg axoplasm ml⁻¹ was homogenised in buffer and incubated with (*g*) 1 mM, (*h*) 5 mM and (*i*) 20 mM CaCl₂, then boiled in 1% SDS, 1% 2-mercaptoethanol, to terminate digestion.

axoplasm[10]. A similar proteolytic activity may be involved in the Ca^{2+}-sensitive retrograde degeneration of vertebrate axons, in which a transition from filamentous to flocculent forms also occurs[11,12].

Cytochrome c causes the formation of remarkably ordered aggregates of *Myxicola* neurofilaments (Fig. 3). Fresh, or washed, or reconstituted neurofilaments align side by side to form neurofibril-like structures, which frequently terminate in rings. The rings are similar in size and shape to the neurofibrillar rings found *in vivo*, at synapses, and near cut axons[6,13-15]. Rings lacking stems, figure eights and more complex forms are also synthesised *in vitro*, resembling the range of forms found *in vivo*[13].

In summary, *Myxicola* neurofilaments resemble those of both vertebrates and invertebrates in their appearance in electron microscope sections, and in their neurofibrillar and ring-like assemblies. Different treatments, however, cause marked changes in *Myxicola* neurofilament appearance, which we tentatively identify with different states of aggregation of fibrous subunits. The protein molecule(s) involved have not yet been identified, but their component polypeptide chains give a simple and consistent pattern on SDS gels. The major chains (152,000 and 160,000 molecular weights) are rapidly cleaved by an endogenous, Ca^{2+}-activated protease, to give fragments roughly the size of the chains isolated from squid (70,000–90,000 (ref. 3)) and calf (~58,000 (ref. 4)) neurofilaments. But squid axoplasm has no major heavy chains which comigrate with those from *Myxicola*. Therefore present evidence suggests that neurofilaments from different species consist of different polypeptide chains, though possible proteolytic degradation of a common subunit cannot be ruled out.

We thank John Pacy, Brian Kirkham, and John Hopkins for help with the electron microscopy; Peter Knight and Alan Outen for molecular weight standards; Zolly Gabor for preparing the plates, and the director and staff of the Marine Biological Laboratory, Plymouth, for supplying live squid. This work was supported by the Medical Research Council.

DAVID S. GILBERT
BARBARA J. NEWBY*

MRC Cell Biophysics Unit,
Biophysics Department, King's College,
26/29 Drury Lane, London WC2B 5RL, UK

BRIAN H. ANDERTON

School of Engineering and Science,
Polytechnic of Central London,
115 New Cavendish Street,
London W1M 8JS, UK

*Also at Polytechnic of Central London, London W1M 8JS, UK.

1 Wuerker, R. B., and Kirkpatrick, J. B., *Int. Rev. Cytol.*, 33, 45–75 (1972).
2 Davison, P. F., and Taylor, E. W., *J. gen. Physiol.*, 43, 801–823 (1960).
3 Huneeus, F. C., and Davison, P. F., *J. molec. Biol.*, 52, 415–428 (1970).
4 Shelanski, M. L., Albert, S., De Vries, G. H., and Norton, W. T., *Science*, 174, 1242–1245 (1971).
5 Davison, P. F., and Winslow, B., *J. Neurobiol.*, 5, 119–133 (1974).
6 Gilbert, D. S., *Nature new Biol.*, 237, 195–198 (1972).
7 Day, W. A., and Gilbert, D. S., *Biochim. biophys. Acta*, 285, 503–506 (1972).
8 Maxfield, M., *J. gen. Physiol.*, 37, 201–216 (1953).
9 Hodgkin, A. L., and Katz, B., *J. exp. Biol.*, 26, 292–294 (1949).
10 Orrego, F., *J. Neurochem.*, 18, 2249–2254 (1971).
11 Schlaepfer, W. W., *Brain Res.*, 69, 203–215 (1974).
12 Hallpike, J. F., and Adams, C. W. M., *Histochem. J.*, 2, 559–578 (196*I*).
13 Cajal, S. R., *Degeneration and Regeneration of the Nervous System*, 1, (Hafner, New York, 1959).
14 Boycott, B. B., Gray, E. G., and Guillery, R. W., *Proc. R. Soc.*, B'54, 151–172 (1961).
15 Potter, H. D., and Hafner, G. S., *J. comp. Neurol.*, 155, 409–423 ('_ /4).
16 Weber, K., and Osborn, M., *J. biol. Chem.*, 244, 4406–4412 (1969'.
17 Gilbert, D. S., *J. Physiol., Lond.*, (in the press),

Persistent innervation of mammalian sympathetic neurones by native and foreign fibres

CONSIDERABLE evidence has been advanced to support the view that the reinnervation of adult mammalian autonomic

neurones is specific[1-4]. Moreover, although foreign nerve fibres by themselves readily form synaptic connections with denervated ganglion cells[5-11], when native fibres are also present their terminals are thought to be preferred to foreign axon terminals, which are eventually rejected[4]. I have re-examined the ability of neurones in the mammalian superior cervical ganglion (SCG) to select between native and foreign nerve fibres during reinnervation using the technique of intracellular recording. An unexpected result was that many neurones were initially reinnervated by both native and foreign nerves, and retained their dual innervation for at least 10 months.
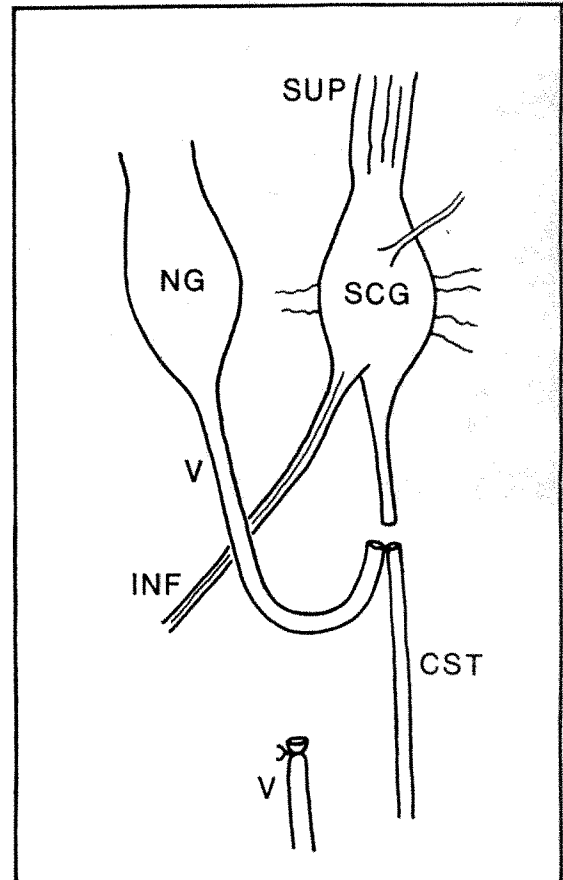
Fig. 1 Diagram of operative procedure (ventral view). NG, nodose ganglion; V, vagus nerve; SUP, superior postganglionic nerve bundle; INF, inferior postganglionic nerve bundle. Drawing not to scale.

The right cervical sympathetic trunk (CST) in 17 adult albino guinea pigs (200–400 g) was aseptically exposed under pentobarbital anaesthesia, and cut 3–5 mm below the lower pole of the SCG. The right vagus nerve was also cut about 2–3 mm caudal to this level, and its proximal end laid side by side with the proximal end of the CST (Fig. 1); the distal end of the vagus nerve was then ligated and the wound closed. After intervals ranging from 23 d to 10 months, the SCG was removed together with suitable lengths of the CST, the vagus nerve, and the superior postganglionic nerve, and placed in a perfusion chamber. In 14 animals, both the CST and the vagus nerve entered the neuroma which had formed at the nerve anastomosis; in three animals the vagus nerve did not enter the neuroma, and these ganglia were not considered further. The CST and the vagus nerve proximal to the anastomosis, and the superior postganglionic branch (Fig. 1), were taken into close fitting suction electrodes for stimulation and recording. Neurones within the ganglion were impaled with glass microelectrodes[12] and their responses to CST and vagal stimulation recorded.

By 23 d after the initial surgery, most impaled neurones
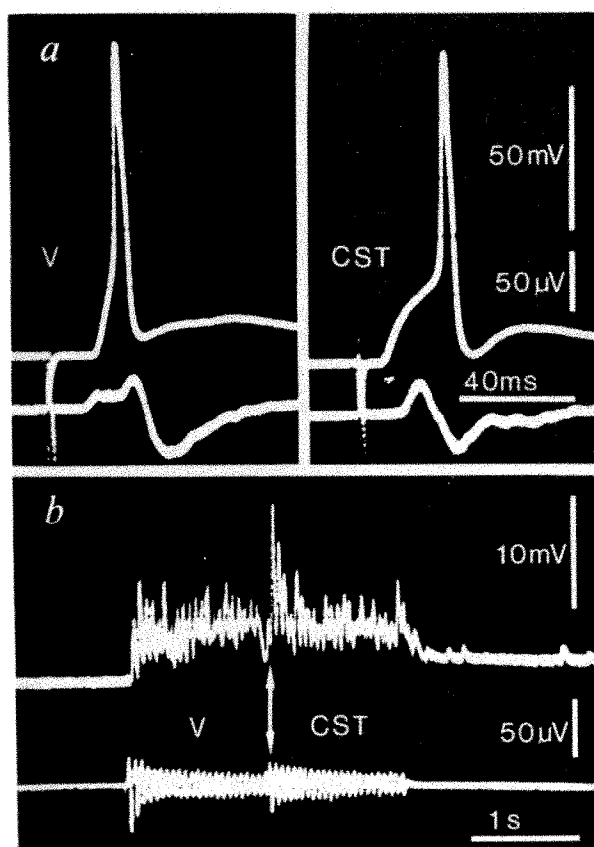
Fig. 2 *a*, Dually innervated neurone impaled 273 d after initial operative procedure. Upper traces show that suprathreshold responses are elicited following stimulation of both the native (CST) and vagus (V) nerves. Lower trace is an extracellular recording from the superior postganglionic nerve bundle. *b*, Upper trace shows that repetitive stimulation (20 s^{-1}) of the vagus nerve causes a depression of transmission in a dually innervated neurone impaled 291 d after initial operative procedure, but does not affect the response to subsequent stimulation of the CST (arrow). This effect can also be seen in the compound action potentials recorded from the superior postganglionic nerve bundle (lower trace). The intracellularly recorded effect was clearer when, as in this cell, the e.p.s.p.s elicited were subthreshold and thus not obscured by action potentials.

were found to be reinnervated. In about half the neurones excitatory postsynaptic potentials (e.p.s.p.s) were elicited after stimulation of only the CST or the vagus nerve; in the remainder, however, e.p.s.p.s were recorded in response to stimulation of both nerves. In a series of 101 neurones impaled in eight ganglia 23 d to 3 months after nerve section, synaptic potentials could be evoked in all but three cells; 60 neurones (59.4%) had e.p.s.p.s after both CST and vagal stimulation and, in many of these cells (28.3%), the response was above threshold for either nerve (Fig. 2*a*). That these neurones were influenced by two separate sets of nerve terminals was shown by summation of the e.p.s.p.s when the two nerves were stimulated at an interval adjusted so that the e.p.s.p.s elicited from both sources occurred simultaneously. Furthermore, depression of transmission caused by repetitive stimulation of one pathway did not depress transmission caused by stimulating the other nerve (Fig. 2*b*). Of the neurones which received innervation from a single source, about half were innervated by the CST, and half by the vagus nerve.

After 6–7 months, 19 out of 41 neurones (46.3%) impaled in two ganglia were found to be dually innervated, whereas after 8–10 months, 42 out of 78 neurones (53.8%) in four ganglia could be shown to receive endings from both sources. Thus little change occurred in the proportion of dually innervated cells over a 10 month period. There was also no obvious tendency for CST synapses to predominate

over vagal terminals: even after 8–10 months the portion of neurones having suprathreshold responses to both vagal and cervical trunk stimulation (Fig. 2*a*) was about one-third. Indeed, a greater number of neurones were innervated by vagal fibres than CST fibres after 8–10 months, although after 23 d to 3 months the number of cells innervated from each source was nearly equal.

These results should not be taken to indicate that connections within the SCG are not normally highly specific[1-5], or that ganglion cells may not have some ability to select correct synaptic connections in other circumstances. It may be, for example, that the large number of vagal fibres which grow into the ganglion overwhelm some more subtle selective mechanism. In the conditions of these experiments, however, mammalian sympathetic neurones show little or no preference for native or foreign fibres during reinnervation, and, over a period of 10 months, have no obvious tendency to reject inappropriate synaptic connections. This latter finding is similar to a recent study of dually innervated muscle fibres in mammals[13] (see also ref. 14). Thus the adult mammalian nervous system seems less capable of selectivity during reinnervation than has previously been thought, and in this regard seems to be quite different from the nervous systems of lower vertebrates in which this ability is clearly present (see, for example, refs 15 and 16).

This work was supported by the NIH.

Dale Purves

Department of Physiology and Biophysics,
Washington University Medical School,
660 South Euclid Avenue,
St Louis, Missouri 63110

1 Langley, J. N., *J. Physiol., Lond.,* **18**, 280–284 (1895).
2 Langley, J. N., *J. Physiol., Lond.,* **22**, 215–230 (1897).
3 Murray, J. G., and Thompson, J. W., *J. Physiol., Lond.,* **135**, 133–162 (1957).
4 Guth, L., and Bernstein, J. J., *Expl Neurol.,* **4**, 59–69 (1961).
5 Langley, J. N., *J. Physiol., Lond.,* **23**, 240–270 (1898).
6 Langley, J. N., and Anderson, H. K., *J. Physiol., Lond.,* **30**, 439–442 (1904).
7 Langley, J. N., and Anderson, H. K., *J. Physiol., Lond.,* **31**, 365–391 (1904).
8 De Castro, F., *Trab. Lab. Invest. Biol. Univ. Madr.,* **34**, 217–262 (1942).
9 Guth, L., *Am. J. Physiol.,* **185**, 205–208 (1956).
10 Matsumura, M., and Koelle, G. B., *J. Pharmac. exp. Ther.,* **134**, 28–46 (1961).
11 McLachlan, E., *J. Physiol., Lond.,* **237**, 217–242 (1974).
12 Purves, D., *J. Physiol., Lond.,* (in the press).
13 Frank, E., Jansen, J. K. S., Lømo, T., and Westgaard, R., *Nature,* **247**, 375–376 (1974).
14 Scott, S. A., *Science* (in the press).
15 Landmesser, L., and Pilar, G., *J. Physiol., Lond.,* **211**, 203–216 (1970).
16 Attardi, D. G., and Sperry, R. W., *Expl Neurol.,* **7**, 46–64 (1963).

Sex difference in ontogenesis of circadian adrenocortical rhythm in cortisone-primed rats

The hormonal milieu in the neonatal period has profound influences on the morphological, biochemical and functional development of the brain[1,2]. Krieger reported that the administration of corticosteroid (dexamethasone or hydrocortisone) to 2–4-d-old rats suppressed the circadian rhythm of plasma corticosterone at 30 d of age[3], whereas treatment at 12–14 d of age did not affect the rhythmicity. We have investigated whether the suppression of corticosterone rhythm results in its permanent derangement throughout adult life or merely indicates its delayed appearance. After this paper was submitted, Krieger published the results of a similar study[4].

Litters of Wistar rats were reared in a room with 12-h light and 12-h dark cycle (lights on 0600–1800). Within 72 h after birth, all pups were injected subcutaneously with either 0.5 mg of cortisone acetate (Upjohn Co., Kalamazoo, Michigan) or an equal volume of saline. Rats were weaned at day 27, group-housed by sex and treatment, and handled every day. Food and water were available *ad libitum*. On

Table 1 Effect of neonatal cortisone administration on morning and evening levels of plasma corticosterone

Treatment	Age (d)	Plasma corticosterone (μg dl^{-1}, mean ± s.e.)			
		Male		Female	
		0800	2000	0800	2000
Control	32	11.2±0.9 (8)	33.1±2.3*(8)	12.8±1.1 (8)	36.2±2.3*(8)
	42	10.1±0.4 (6)	39.5±2.8*(6)	13.8±1.7 (6)	43.7±2.0*(6)
	55	8.4±1.5 (10)	38.4±1.8*(10)	15.8±1.4 (10)	47.5±2.9*(10)
	95	15.9±1.4 (5)	42.0±3.4*(7)	16.1±2.8 (6)	50.3±5.1*(6)
Cortisone	32	12.2±0.9 (8)	15.1±1.0 (8)	14.0±1.0 (8)	15.0±1.4 (8)
	42	11.6±0.9 (6)	13.3±1.7 (6)	15.7±1.4 (6)	24.3±2.0*(6)
	55	9.7±0.9 (10)	11.3±1.4 (10)	16.1±1.6 (10)	40.5±2.5*(10)
		7.9±1.3 (6)†	12.7±1.4 (6)†	12.1±0.5 (6)†	34.9±2.7*(6)†
	95	11.9±0.6 (5)	13.0±1.0 (7)	—	—
	130	12.3±1.4 (6)†	22.7±0.3*(6)†	19.3±3.2 (6)†	49.7±3.5*(6)†
		6.8±1.0 (8)	33.8±2.6*(8)	16.3±1.5 (8)	48.5±4.3*(8)

*$P<0.01$ against 0800 by Mann–Whitney U test.
†Data derived from the second series of experiments (see text).
Numbers of animals given in parentheses

days 32, 42, 55, 95 and 130, animals were decapitated at either 0800 or 2000 and trunk blood was collected for plasma corticosterone determination[5]. At each sampling time, the experimental groups were arranged so that each group represented a cross section of the litters available. A preliminary study indicated that these two time points gave the trough (0800) and the peak (2000) of plasma corticosterone rhythm in the condition used. On day 55, additional males were tested for plasma corticosterone response to ether stress at 1000. Animals were exposed to ether for 1.5 min and blood was obtained 15 min after the onset of stress[6]. In the other series of experiments, all animals were treated neonatally with cortisone and housed in the same way. On days 55 and 95, the profile of plasma corticosterone over a 24-h period was studied by blood sampling at 6-h intervals starting at 0800.
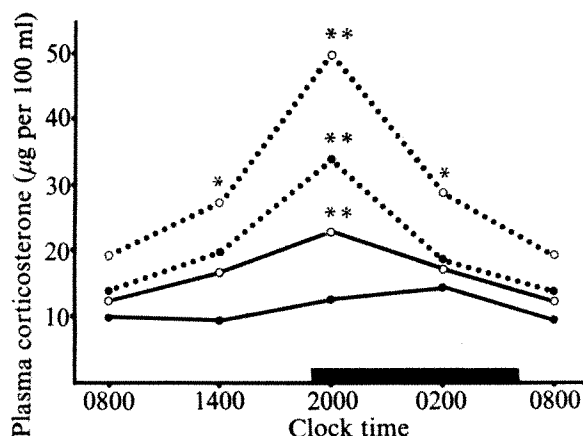
Fig. 1 Circadian variations of plasma corticosterone in cortisone-treated rats. Each data point represents the mean of six animals. ——, Male rats; •••••, female rats; ●, 55 d of age; ○, 95 d of age. *, $P<0.05$; **, $P<0.01$, compared with 0800 value (Mann–Whitney U test). Dark period is shown as black horizontal bar.

The day–night variation of plasma corticosterone level was apparent from 32 d of age in the control rats of both sexes. Neonatal cortisone administration did not change the 0800 level significantly, but suppressed the rise of the 2000 level completely at 32 d of age in both sexes. At 42 d of age, however, the cortisone-treated female rats showed a small but definite nocturnal rise of plasma corticosterone. At 55 d of age, and thereafter, the adrenocortical rhythm was completely restored. In contrast, the effect of exposure to cortisone was more marked and lasted longer in the male rats. The plasma corticosterone rhythm was absent on day 55, equivocal or diminished on day 95 but ultimately established on day 130 (Table 1). No phase shift in circadian

rhythm was induced by the treatment (Fig. 1). The fact that the response of plasma corticosterone to ether stress was not impaired (Table 2) supports the concept that basal circadian rhythm and stress-induced change in the hypothalamo–pituitary–adrenal axis are regulated by somewhat different mechanisms[7]. Although corticosterone response to ether stress has been reported to have circadian variation[8], we did not test whether neonatal cortisone treatment suppressed such rhythm of stress responsiveness.

Table 2 Response of plasma corticosterone to ether stress in 55-d-old male rats treated neonatally with cortisone

Treatment	Plasma corticosterone (μg dl^{-1}, mean±s.e.)	
	Basal	Stress
Control	7.7±1.0 (6)	50.4±5.0*(6)
Cortisone	11.2±1.5 (6)	47.4±4.5*(6)

*$P<0.01$ against basal by Mann–Whitney U test.
Numbers of rats given in parentheses.

Sex difference in the vulnerability of corticosterone rhythm to neonatal cortisone administration was rather unexpected. In Krieger's study[4], the hydrocortisone-treated rats of both sexes, were reported to have 'normal' corticosterone rhythm at 80 d of age. Since data on earlier days are lacking, the results do not necessarily mean the same recovery course of corticosterone rhythm for both sexes. Furthermore, Krieger's figures show that the mean plasma corticosterone level at 2000 in the hydrocortisone-treated male rats was about half of that in the control male rats. The failure to find a significant difference between the two values might have resulted from the number of animals studied (only three rats at each time point) or a relatively large variation in the corticosterone level at 2000, as shown in our data on 95-d-old male rats treated with cortisone. The results of the two studies are not, therefore, mutually exclusive.

Concerning the sex difference of adrenocortical periodicity, Critchlow et al. have shown that a higher resting level and marked circadian fluctuation of plasma corticosterone are associated with the presence of mature ovary[9]. It has also been shown that a rather marked enhancement in the amplitude of circadian corticosterone rhythm takes place at puberty in the female, and that ovariectomoy attenuates the change[10,11]. Although adrenal–gonadal interactions are multiple and complex[12], a marked sex difference in the circadian rhythm of hypothalamic corticotrophin-releasing factor content and its phase shift by ovariectomy, reported by Hiroshige et al.[13], provide good evidence that gonadal hormones can modulate the pituitary–adrenal periodicity through a central mechanism.

In rats and in many other species, sexual differentiation

of the brain is initiated in neonatal life. The male pattern is programmed by the presence, and female pattern by the absence, of androgen during a relatively short period of infancy known as the critical period[14]. This 'program' is stored for a while and expresses itself at puberty. It is therefore important to study whether the sex difference in the ontogenesis of plasma corticosterone periodicity observed in rats primed with cortisone in infancy is related to event(s) in early sexual differentiation or rather to factor(s) associated with the onset of puberty.

SUSUMU MIYABO
TOMOICHIRO HISADA

Department of Medicine,
Kanazawa University,
Kanazawa, Japan

1 Schaprio, S., in *Influence of Hormones on the Nervous Systems* (edit. by Ford, D. H.), 63–73 (S. Karger, Basel, 1971).
2 Vernadakis, A., and Woodbury, D. M., *ibid.*, 85–97.
3 Krieger, D. T., *Science*, 178, 1205–1207 (1972).
4 Krieger, D. T., *Neuroendocrinology*, 16, 355–363 (1974).
5 Givner, M. L., and Guy Rochefort, J., *Steroids*, 6, 485–489 (1965).
6 Davidson, J. M., Jones, L. E., and Levine, S., *Endocrinology*, 82, 655–662 (1968).
7 Krieger, D. T., in *Biological Rhythms and Endocrine Function* (edit. by Hedlund, L. W., Franz, J. M., and Kenny, A. D.), 169–184 (Plenum, New York, 1975).
8 Gibbs, F. P., *Am. J. Physiol.*, 219, 288–292 (1970).
9 Critchlow, V., Lieberlt, R. A., Bar-Sela, M., Moutcastle, W., and Lipscomb, H. S., *Am. J. Physiol.*, 205, 807–815 (1963).
10 Ramaley, J. A., *Steroids*, 20, 185–197 (1972).
11 Ramaley, J. A., *Steroids*, 21, 597–608 (1973).
12 Kitay, J. I., in *Functions of The Adrenal Cortex* (edit. by McKerns, K. W.), 775–811 (Appleton–Century–Crofts, New York, 1968).
13 Hiroshige, T., Abe, K., Wada, S., and Kaneko, M., *Neuroendocrinology*, 11, 306–320 (1973).
14 Barraclough, C. A., in *Neuroendocrinology* (edit. by Martini, L., and Ganong, W. F.), 61–99 (Academic, New York, 1967).

Oestrogen-responsive human breast cancer in long term tissue culture

STUDIES of steroid hormone dependence of human breast cancer have been hampered by a lack of a suitable *in vitro* model system which can reproduce the responses of human breast cancer observed *in vivo*. We now report the characterisation of several human breast cancer cell lines, at least one of which shows marked dependence on oestrogens for growth. This oestrogen dependence seems to be similar in a variety of ways to that observed clinically *in vivo*.

MCF7 cells, a cloned line of human breast cancer, have been partially characterised[1]. This line was found by a specific enzymatic assay in which the synthesis of lactose was confirmed by chromatography of the products (M.E.L. and B. V. Vonderhaar, unpublished), to synthesise α lactalbumin, and formed 7.05 pmol lactose per 30 min per μg protein. Other breast cancer cell lines used—HT39, Kielty and G11—were derived from malignant effusions in patients with metastatic breast cancer. Under the electron microscope, these cell lines appear epithelioid with numerous microvilli, desmosomes, some rough endoplasmic reticulum and Goldi apparatus.

As physiological concentrations of steroid hormones are present in the serum containing media in which the cells are usually grown[6] and as these endogenous hormones may obliterate responses to additional hormones, the serum was stripped of most endogenous steroid by repeated mixing with dextran-coated charcoal at 55 °C for 45 min[7]. Removal of oestradiol was monitored by the addition of a trace amount of tritiated oestradiol at the beginning of the procedure and after the decrease in radioactivity, which was reduced to less than 1% after successive charcoal treatments. Incubations of cells in various hormone combinations were carried out at least in triplicate. After various incubation periods the cells were pulsed for 1 h with labelled amino acids or nucleosides, collected and macromolecular synthesis measured[8].

Table 1 shows the effects of oestradiol on DNA synthesis

in the MCF7 cell line. Oestradiol 10^{-8} M more than doubles nucleoside incorporation in the experiment shown. We noted reproducible increases in macromolecular synthesis with as little as 10^{-11} M oestradiol. If oestradiol (10^{-9}–10^{-7} M) is added to cells in untreated serum no augmentation of macromolecular synthesis is noted. Oestradiol stimulation of these cells shows a clear-cut maximum at concentrations of 10^{-10}–10^{-7} M oestradiol. The concentration of oestradiol which half saturates the oestradiol receptor *in vitro* closely corresponds to that concentration of oestradiol which half-maximally stimulates the cells, as determined by competitive protein-binding assay using dextran-coated charcoal (see below). Higher concentrations of oestradiol are less effective and concentrations of about 2×10^{-6} M clearly inhibit the cells below control levels (Table 1). Diethylstilboestrol, which stimulates these cells as effectively as oestradiol, also inhibits the cells at more than 5×10^{-7} M.

Table 1 Effects of oestradiol, diethylstilboestrol (DES) and Tamoxifen on DNA synthesis

Condition	Thymidine incorporation
Control	18 ± 4.3
Oestradiol 10^{-8} M	41 ± 5.2
Oestradiol 5×10^{-6} M	6 ± 2.6
DES 10^{-8} M	43 ± 3.1
DES 5×10^{-6} M	5 ± 1.7
Tamoxifen 10^{-7} M	4 ± 1.6
Tamoxifen 10^{-7} M + oestradiol 10^{-8} M	26 ± 4.8

Cells growing in log phase were collected and plated in quadruplicate at uniform density in Eagle's MEM supplemented with 10% foetal calf serum, and 2 × glutamine. After 24 h the medium was replaced with MEM containing 2% serum from which endogenous steroids were removed by stirring for 45 min with dextran-coated charcoal at 55 °C twice. The medium was sterilised by filtration. Various hormones were added to the medium as 1,000 × concentrates in ethanol as shown in Fig. 1. After 48 h 0.5 μCi ³H-thymidine (New England Nuclear, 40 Ci mmol⁻¹) was added to each dish. One hour later the dish was washed with phosphate-buffered saline twice and the cells collected after detachment with trypsin–EDTA solution. The cell buttons were suspended in ice water and sonnicated in a Brownson sonnicator for 4 s at the lowest setting. Aliquots were used for determination of protein by the method of Lowry[13] and for TCA precipitation. Acid-insoluble counts were collected on 0.45 μm Millipore filters and counted in a liquid scintillation counter (efficiency 35%). Values (d.p.m. × 10^{-3} per μg protein per h) are averages of quadruplicate determinations ±1 s.d.

This biphasic response to oestrogen suggests that the same population of cells which is stimulated by oestradiol at lower concentrations of oestrogen may be inhibited at higher concentrations. As such concentrations are readily achieved in patients with metastatic breast cancer treated with large doses of oestrogen, our data suggest that this regimen may be effective because the tumour itself is directly inhibited by the oestrogen rather than by an indirect effect of the oestrogen on some other trophic hormone. This is an attractive explanation for the seemingly paradoxical stimulation of some tumours by oestrogen at physiological levels and tumour regression seen at higher doses of administered oestrogens[9]. To demonstrate more clearly the oestrogen dependence of these cells, we used the anti-oestrogen ICI46,474 (Tamoxifen), which is a substituted hexoestrol derivative[10]. Figure 1 shows that 10^{-7} M Tamoxifen depressed DNA synthesis below control levels: this effect could be overcome by simultaneous addition of 10^{-8} M oestradiol. Hundredfold lower concentrations of oestradiol were sufficient to overcome Tamoxifen inhibition of macromolecular synthesis. Similar effects were noted when incorporation of ¹⁴C-leucine into protein was measured.

Figure 1 shows the time course of the response to both oestradiol and Tamoxifen. At varying times after the addition of Tamoxifen to the cells, isotopically labelled pre-
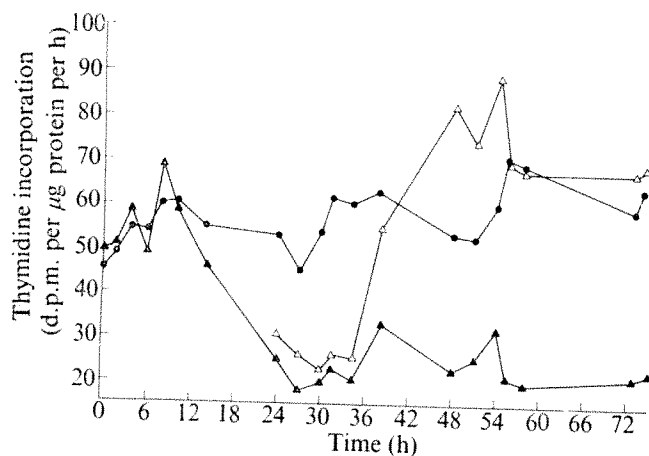
Fig. 1 Kinetics of Tamoxifen inhibition and oestradiol stimulation of human breast cancer *in vitro*. Cell plating and collection, see Table 1. ●, Control cells; ▲, 10^{-7} M Tamoxifen-treated cells. After 24 h, 10^{-8} M oestradiol was added to an additional set of dishes without removing the Tamoxifen (△). s.d. for each point was usually less than 10%.

cursors were added for 1 h for determination of net synthetic rate at that time point. By about 10 h, 10^{-7} M Tamoxifen begins to strongly inhibit macromolecular synthesis which falls to about one-third of control levels by 24 h and remains at this level throughout the duration of the experiment. If at 24 h, 10^{-8} M oestradiol is added to some of the Tamoxifen-containing dishes without removing the Tamoxifen, the inhibition is shown to be completely reversible with recovery beginning about 10 h after addition of the oestradiol. Similar changes are noted when protein synthetic rates are measured. We have observed a rise in thymidine incorporation rates to values which exceed control levels for several hours during the early period of oestrogen stimulation of Tamoxifen-inhibited cells. This suggests that some degree of synchronisation of the cells may have occurred during the incubation in Tamoxifen alone, with a larger cohort of cells entering the DNA synthetic phase of the cell cycle after oestradiol rescue. If a cloned line of the hormone-responsive MCF7 cells are left in Tamoxifen alone, most cells begin to round up, detach from the surface and die after about 4 d although occasional colonies of 'normal' Tamoxifen-resistant and possibly hormone-independent cells are sometimes noted. The phenomenon of Tamoxifen killing is invariably reversible if oestradiol is added to the medium by 48 h even though the anti-oestrogen remains in the medium.

We have duplicated these experiments in serum-free conditions. In spite of the total lack of oestrogen in the medium, Tamoxifen still inhibits the cells below control levels. The possibility that Tamoxifen-oestrogen receptor complexes are binding to chromatin sites leading to decreased transcription of regulatory RNA segments below control levels is under investigation.

That Tamoxifen is inhibiting these cells primarily by interfering with the trophic effects of oestrogen is suggested by three lines of reasoning. First, Tamoxifen inhibition is reversible by the addition of oestradiol. Simultaneous addition of Tamoxifen and oestradiol leads to stimulation if the Tamoxifen is less than 2 logs higher in concentration than oestradiol. Second, in other experiments, Tamoxifen had no effect on cells in culture not responsive to oestradiol, such as hepatoma tissue culture cells, HeLa cells, or the HT39, Kielty and G11 breast lines. Third, Tamoxifen is capable of binding to the same high affinity oestrogen receptor molecules found in the cytoplasm of these cells. Using a dextran-coated charcoal assay[11] one of these cell lines, MCF7, was shown to contain about 70 femtomol oestradiol receptor per mg cytoplasmic protein with an apparent K_d of about 5×10^{-11} M, a value in reasonable agreement with

a previously published value[12]. Tamoxifen binds to this receptor with about a 1,000-fold lower affinity than oestradiol, explaining in part the ability of 10^{-8} M oestradiol to stimulate the cells in the presence of 10^{-7} M Tamoxifen. In fact, the dose–response curve of the inhibition of these cells by Tamoxifen closely approximates the displacement curve of oestradiol from its cytoplasmic receptor by Tamoxifen.

The MCF7 line has been maintained in continuous tissue culture for 2 yr but still demonstrates remarkable oestrogen dependence. The potential value of a hormone-dependent human breast cancer in long term tissue culture for the study of the mechanism(s) by which steroid hormones exert their trophic effects is significant, particularly in view of the likelihood of obtaining regulatory variants or mutants which are hormone independent. In addition these cells may prove useful in the analysis of oestrogen action in non-malignant tissue, as oestrogen-responsive cell systems in continuous tissue culture have not been widely reported.

The Kielty, G11, and HT39 lines are not stimulated by 10^{-8} M oestradiol nor are they inhibited by Tamoxifen. They are, however, killed by 10^{-5} M oestradiol. The specificity of this response is being investigated.

In results to be reported elsewhere, two other human breast cell lines, Evsa T and Evsa E developed in our laboratory, both respond to 10^{-8} M oestradiol with the accumulation of α lactalbumin without a significant increase in general macromolecular synthesis[1].

Marc E. Lippman
Gail Bolan

Medical Breast Cancer Section,
Medicine Branch, National Cancer Institute,
Bethesda, Maryland 20014

1 Soule, H. D., Vazquez, J., Long, A., Abert, S., and Brennan, M., *J. natn. Cancer Inst.*, 51, 1409–1416 (1973).
2 Young, R. K., Cailleau, R. M., Mackay, B., and Reeves, W. J., *In Vitro*, 9, 239–245 (1974).
3 Cailleau, R., Young, R., Olive, M., and Reeves, W., *J natn. Cancer Inst.*, 53, 661–674 (1974).
4 Plata, E. J., Aoki, T., Robertson, D. D., Chu, E. W., and Gerwin, B. I., *J. natn. Cancer Inst.*, 50, 849–857 (1973).
5 Gewant, W. C., and Goldenberg, I. S., *Eur. Surg. Res.*, 2, 392–400 (1970).
6 Esber, H., Payne, I., and Bogden, A., *J. natn. Cancer Inst.*, 50, 559–562 (1973).
7 Armelin, H. A., Nishikawa, K., and Sato, G. H., in *Control of Proliferation Animal Cells* (edit. by Clarkson, B., and Baserga, R.), 97 (Cold Spring Harbor Laboratory, 1974).
8 Lippman, M. E., Halterman, R. M., Leventhal, B. G., Perry, S., and Thompson, E. B., *J. clin. Invest.*, 52, 1715–1722 (1973).
9 Bulbrook, R. D., in *Breast Cancer: Early and Late*, 51 (Year Book, Medical Publishers, Chicago, 1970).
10 Anstall, H. B., in *Biochemistry of Hormones* (edit. by Rickenberg, H. V.), 243 (University Park Press, Baltimore, 1974).
11 McGuire, W. L., and De la Garza, M., *J. clin. Endocr. Metab.*, 36, 548–552 (1973).
12 Brooks, S.C., Locks, E. R., and Soule, H. D., *J. biol. Chem.*, 248, 6251–6253 (1973).
13 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randal, R. J., *J. biol. Chem.*, 193, 265–279 (1951).

Non-encephalitogenic antigen-induced suppression and reversal of allergic encephalomyelitis

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease of the central nervous system[1] induced in experimental animals by the myelin basic protein (BP)[2] or specific peptide regions derived from the BP[3] emulsified with Freund's complete adjuvant (FCA).

Previous studies have shown that the encephalitogenic tryptophan region of BP, H–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Lys–OH (peptide S3) induces delayed type hypersensitivity (DTH) specific for peptide S3 and BP (ref. 4). The DTH determinant was separated from that which produces disease and shown to overlap the C-terminal sequence of peptide S3

Table 1 Suppression and treatment of allergic encephalomyelitis with non-encephalitogenic peptide S42

Group no.	Challenging antigen (µg per FCA)	Treatment FIA	Clinical EAE	Reversal clinical EAE	Histological EAE
1	25 µg S42	None	0/15	None	0/15
2	100 µg S42	None	0/10	None	0/15
3	5,000 µg S42	None	0/10	None	0/10
4	100 µg BP	1 mg S42	2/4	2/4	2/4
5	100 µg BP	FIA	4/4	0/4	4/4
6	150 µg BP	2.5 mg S42	5/5	5/5	2/5
7	150 µg BP	FIA	5/5	0/5	5/5
8	150 µg BP	saline	6/6	0/6	6/6
9	150 µg BP	4.0 mg S42	7/7	6/7	4/7
10	150 µg BP	1 mg BP	4/4	2/4	3/4
11	150 µg BP	FIA	7/7	0/7	7/7

The guinea pigs were challenged with Freund's complete adjuvant (FCA) emulsion of either peptide S42 or bovine basic protein. Groups 1, 2 and 3 received no treatment. For groups 4 and 5, the suppressive treatment in Freund's incomplete adjuvant (FIA) was started on day 8, before the appearance of clinical signs of experimental allergic encephalomyelitis (EAE) and was continued through day 17. For groups 6, 7 and 8, the treatment began the day hind leg paralysis was observed and was continued for 10 consecutive days. For groups 9, 10 and 11, the treatment was started two days after hind leg paralysis was observed and was continued for 10 consecutive days. Surviving animals were killed on day 32 for histological examination of the brain and spinal cord tissue.

(ref. 5). Further, the immunological response of sensitised animals to the EAE or to the DTH determinant could not be distinguished clinically or histologically from similar responses to the intact BP molecule. We have shown[6,7] that the encephalitogenicity of the tryptophan region is destroyed by amino acid substitutions or deletions from the sequence of peptide S3, but this treatment did not abolish the DTH properties of the resulting antigens. Deletion of the Gly–Ala–Glu–Gly sequence gives rise to peptide S17, H–Phe–Ser–Trp–Gln–Lys–OH, previously shown to induce and elicit DTH responses similar to those obtained for peptide S3. Further, animals sensitised to peptide S3, S17 or the BP exhibit DTH responses which were elicited by either of the three antigens[7]. These results indicate that the non-encephalitogenic antigen is recognised by cells sensitised to the EAE-inducing antigen and thus may be useful in the treatment of this cell-mediated disease. Peptide S42 was prepared by the Merrifield solid phase synthesis technique[8] to contain four linearly spaced repeating units of S17. Approximately 700 mg of peptide S42 was prepared at one time. Based on 0.25 mEq glycine per gram resin, a recovery of 40–60% was calculated. The purity of peptide S42 was established by chromatography and high voltage and polyacrylamide disc gel electrophoresis[9]. Amino acid analysis[10] of purified peptide S42, H–(Phe–Ser–Trp–Gln–Lys)$_4$–Gly–OH, gave whole integers of expected residues, including 4 mol tryptophan per mol peptide.

The results of experiments designed to study the efficacy of non-encephalitogenic peptide S42 in inhibiting the development (suppression) and in reversing (treatment) signs of EAE are summarised in Table 1. Daily subcutaneous injections of 1 mg peptide S42 starting on day 8 after challenge, significantly reduced the incidence of disease compared with Freund's incomplete adjuvant (FIA) treated controls (compare groups 4 and 5). Daily treatment of animals in group 6 with 2.5 mg peptide S42 for 10 consecutive days was sufficient to reverse tremor and hind leg paralysis in addition to lethargy and weight loss in the five treated animals. Histological examination of the brain and spinal cord tissues on day 32 after challenge revealed the presence of EAE lesions in 2 out of 5 of the animals in spite of the complete reversal of the clinical signs of EAE. Control guinea pigs similarly treated with FIA or saline (groups 7 and 8) died between days 10 and 14 or were killed because of severe paralysis.

Peptide S42-induced reversal of clinical signs of EAE was further studied in an advanced stage of the disease. The treatment for guinea pigs in group 9 was initiated on the second day after the onset of hind leg paralysis. Within 4–6 treatment days, paralysed animals regained the use of their hind legs and were able to run about the cage; however, one of seven guinea pigs died after the second treatment without evidence of improvement in his disease state. In contrast, treatment of

group 10 with 1 mg bovine BP was partially successful in reversing the disease at this late stage of development. None of the FIA-treated controls, group 11, recovered from EAE.

The mechanism of suppression and reversal of disease cannot be elucidated from this study. The primary event of the immune response requires the combination of antigen with receptors on the surface of reactive cells[11]. Cell surface receptors in BP-sensitised animals are specific and recognise the encephalitogenic protein, the tryptophan region and analogous antigens such as peptide S42. This recognition may be described as antigen-induced paralysis of encephalitogenic cells leading to complete reversal of disease. Compared with the effect of anti-lymphocyte serum and cyclophosphamide[12,13], peptide S42 did not induce transient or prolonged lymphopenia, and the treated animals remained immunoresponsive during and after prolonged treatment with peptide S42. Repeated immunisation with peptide S42 failed to induce the formation of antibodies which reacted with BP or with peptide S42.

Studies have shown that intact BP injected before or shortly after the administration of an encephalitogenic dose of the BP reduces the incidence of EAE[14,17]. The use of the BP for treatment, found to be effective in reversing signs of EAE in monkeys, is predicated on uninterrupted administration of the antigen during the critical period of disease[17]. The possibility of *de novo* induction of EAE with the administration of large doses of the encephalitogen should be recognised, however[18]. A synthetic random amino acid copolymer (cop 1) has been used to suppress disease development in guinea pigs[19]. Unlike peptide S42, cop 1 induces the formation of humoral antibodies. Its amino acid sequence is unknown and lacks phenylalanine, serine, tryptophan and glutamine residues known to be essential in the linear sequence necessary for maximum recognition by the EAE inducing cells.

The use of peptide S42 is advantageous in view of its non-encephalitogenic properties and its ability to interact specifically with disease-inducing cells and thus to inhibit the development of EAE and reverse a full-blown stage of disease.

G. A. HASHIM

Department of Surgery and Microbiology,
St Luke's Hospital Center and Columbia University,
New York, New York 10025

[1] Paterson, P. Y., *Adv. Immun.*, **5**, 131–208 (1966).
[2] Eylar, E. H., Salk, J., Beveridge, G. C., and Brown, L. V., *Archs Biochem. Biophys.*, **132**, 34–48 (1969).

3 Hashim, G. A., and Eylar, E. H., *Archs Biochem. Biophys.*, **129**, 645–654 (1969).
4 Hashim, G. A., and Schilling, F. J., *Biochem. biophys. Res. Commun.*, **50**, 589–596 (1973).
5 Hashim, G. A., Hwang, F., and Schilling, F. J., *Archs Biochem. Biophys.*, **156**, 298–309 (1973).
6 Hashim, G. A., and Sharpe, R. D., *Nature*, **255**, 484–485 (1975).
7 Hashim, G. A., and Sharpe, R. D., *Immunochemistry*, **11**, 633–640 (1974).
8 Gutte, B., and Merrifield, R. B., *J. biol. Chem.*, **246**, 1922–1941 (1971).
9 Hashim, G. A., and Eylar, E. H., *Archs Biochem. Biophys.*, **129**, 635–644 (1969).
10 Eylar, E. H., and Hashim, G. A., *Archs Biochem. Biophys.*, **131**, 215–222 (1969).
11 Benacerraf, B., and Daul, W. E., in *Cellular Interaction in the Immune Response* (edit. by Cohen, S., Cudkowicz, G. and McCluskey, R. T.), 298 (Karger, Basel, 1971).
12 Waksman, B. H., Arbuoys, S., and Arnason, B. G., *J. exp. Med.*, **114**, 997–1022 (1961).
13 Paterson, P., in *Immunological Diseases* (edited by Santer, M.), 788 (Little, Brown and Co., Boston, 1965).
14 Alvord, E. C., Jr, Shaw, C., Hruby, S., and Kies, M. W., *Ann. N.Y. Acad. Sci.*, **122**, 333–345 (1965).
15 Cunningham, V., and Field, E. J., *Ann. N.Y. Acad. Sci.*, **122**, 346–355 (1965).
16 Einstein, E., Csejtey, J., Davis, W., and Rauch, H., *Immunochemistry*, **5**, 567–575 (1968).
17 Eylar, E. H., Jackson, J., Rothenberg, B., and Brostoff, S. W., *Nature*, **236**, 74–76 (1972).
18 Hashim, G. A., Sharpe, R. D., Carvalho, E., and Stevens, L. E., *Proc. Soc. exp. Biol. Med.*, **149** (1975).
19 Teitelbaum, D., Meshover, A., Hirshfeld, T., Arnon, R., and Sela, M., *Eur. J. Immun.*, **1**, 242–248 (1971).

Isolation of infectious C-type oncornavirus from human leukaemic bone marrow cells

C-TYPE ONCORNAVIRUSES are associated with neoplasms of the haemopoietic organs in several vertebrate species[1]. Molecular hybridisation studies have revealed the presence of an RNA species in human leukaemias that has some homology with animal C-type oncornaviruses[2,3]. In addition, leukaemic cells contain a reverse transcriptase that proves to be serologically closely related to the enzyme from the woolly monkey C-type oncornavirus[4]. Several claims have been made for the presence of C-type particles in malignant cells or plasma of leukaemic
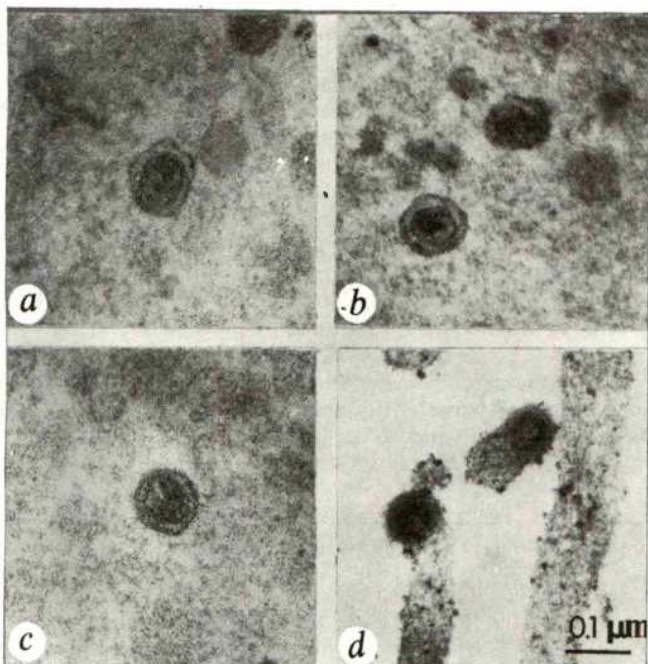
Fig. 1 C-type particles isolated from supernatants of culture fluids. Cleared fluids were spun in cellulose nitrate tubes at 50,000 r.p.m. for 45 min in a Beckman 50 Ti rotor. In the tubes, Millipore membrane filter disks (type 5 VSWP), pore size 25 nm, were positioned on top of supports made of Epon embedding medium. The fluid was mixed with sodium cacodylate-buffered 2% stabilised glutaraldehyde before the run. After the run, the disks were cut into thin oblong strips and immersed in phosphate-buffered 1% osmium tetroxide for 60 min (4 °C). They were then dehydrated in a graded series of isoproponal, treated with toluene, and flat embedded in Epon. Ultrathin sections were made and their contrast enhanced with an aqueous solution of manylacetate and lead citrate. They were examined in a Philips 300 electron microscope. *a*, Particle from phytohaemagglutinin-stimulated bone marrow cells. *b*, Particles from infected human embryonic fibroblasts. *c*, Particle from infected HEK cells. *d*, Budding particles from infected FB289.

patients (for review, see ref. 5). There have also been reports of release of such virus particles in tissue culture lines of leukaemic cells[6,7].

We report here the isolation of a C-type oncornavirus from a 4-yr-old patient with a lymphosarcoma which had progressed to a state of lymphoblastic leukaemia. Bone marrow cells were cultured in liquid suspension in the presence of phytohaemagglutinin (10 μl ml⁻¹, Burroughs, Wellcome, Lot No. 9268). After 3 d culture, the supernatant was examined for the presence of C-type particles using the Millipore filter technique[8] and was found to be positive (Fig. 1).
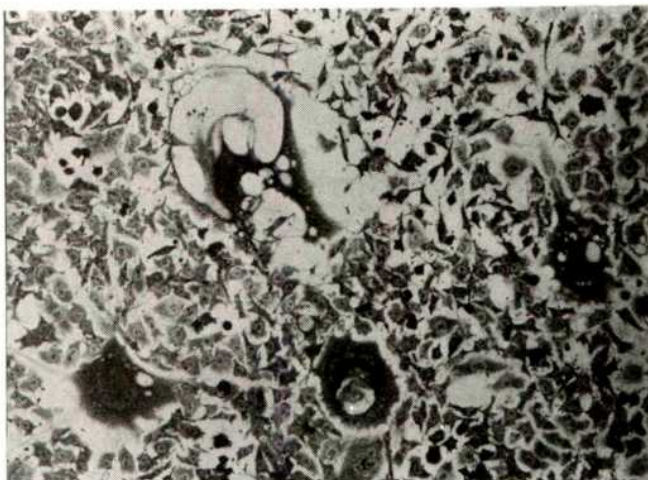
Fig. 2 XC cells with syncytia formed after cocultivation with human leukaemic bone marrow cells. XC cells were grown in the same medium as the bone marrow cells. Cells were grown in Dulbecco's modified MEM supplemented with 2 mM glutamine, 0.2 mM asparagine, 20% of a mixture of equal volumes of foetal bovine serum, normal horse serum, and tryptose phosphate broth containing 100 IU ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. One day after seeding of 2.10⁵ XC cells in 3 ml medium in 50 mm plastic Petri dishes (Greiner, Nurtingen), human bone marrow cells were added in 3 ml medium after removal of the initial medium. Medium was refreshed 3 d later and the dishes were stained with May–Grunwald Giemsa after 1 more d (×105).

Bone marrow cells were seeded on top of a 24-h culture of XC cells. Four days later, conspicuous syncytia were found which were similar to those reported to occur after cocultivation of XC cells and cells infected with a murine leukaemia virus[9] (Fig. 2 and Table 1). In control XC cultures, some small syncytia which contained only a few nuclei were found.

The same cytopathogenic effect was found when fresh leukaemic bone marrow cells were cocultivated directly with XC cells. The production of syncytia persisted on further passage, after trypsinisation of the XC cells which had been in contact with the leukaemic cells.

Cocultivation of XC cells with bone marrow cells from four normal donors, two patients with acute myeloid leukaemia, two with chronic myeloid leukaemia, one with chronic lymphatic leukaemia, one with aplasia and one with secondary polycthaemia vera did not produce syncytia.

The XC cultures which showed a positive cytopathogenic effect released C-type particles, as revealed by electron microscopy (Table 1). Control XC cultures or mixed cultures of XC with normal bone marrow cells were repeatedly negative.

Secondary cultures of human embryonic kidney (HEK) cells were cocultivated with irradiated (9,000 rad X rays) XC cells in their second passage after having been in contact with leukaemic cells. HEK cells proved to replicate the human virus as detected by electron microscopy and the reverse XC cell test[10]. Control experiments with irradiated untreated XC cells did not yield detectable virus. The human embryonic fibroblast line (FB289) exposed to cell-free supernatants of infected HEK cells also replicates the virus.

Table 1 Detection of C-type oncornavirus by mixed cytopathogenicity test with XC cells and electron microscopic examination of culture fluids

Cocultivation of XC cells with:	No. of bone marrow cells plated	No. of syncytia per dish*	Electron microscopy
None	—	17†	—
BALB/c mouse BM infected with RLV	1.0 × 10⁵	1,284	+
Normal human BM	1.0 × 10⁵	10†	
	5.0 × 10⁵	18†	
	1.0 × 10⁶	16†	—
Leukaemic BM, stimulated by phytohaemagglutinin	1.0 × 10⁵	471	+
Leukaemic BM, unstimulated	1.0 × 10⁵	393	+
	2.5 × 10⁵	738	
	5.0 × 10⁵	901	
XC + leukaemic BM, 2nd passage	1.0 × 10⁵	520	+
3rd passage	1.0 × 10⁵	465	
4th passage	1.0 × 10⁵	606	+
XC + normal BM, 3rd passage	1.0 × 10⁵	15†	—
HEK + irradiated XC (+ leukaemic BM), 2nd passage	1.0 × 10⁵	352	+
HEK + irradiated XC	1.0 × 10⁵	18†	—
HEK	1.0 × 10⁵	14†	—
FB289 infected with supernatant from infected HEK cells	1.0 × 10⁵	240	+
FB289	1.0 × 10⁵	13	—

* Each experiment was done in quadruplicate. Petri dishes were coded and syncytia counted blindly and independently by two researchers using an inverted microscope at a magnification of ×75.
† Only small syncytia with less than four nuclei.
BM, Bone marrow.

Indirect immunofluorescence tests were carried out on acetone-fixed cultured cells to assess the serological relationship of this putative human virus isolate to other mammalian C-type oncornaviruses, but also to exclude the possibility of laboratory contamination. At the time when the virus was isolated from the child the only C-type viruses used in our laboratories were murine leukaemia viruses (MuLVs). For that reason several antisera to murine C-type viruses were tested on these cultures (Table 2). Polyvalent rat antiserum to Rauscher murine leukaemia virus (RLV) numbers 45 and 76 have been used to study the presence of C-type oncornaviral antigens in human bone tumour cultures[11]. Their specificity has therefore been extensively tested. They show a broad group-specific reactivity with various ecotropic and xenotropic mouse viruses, endpoint ranging from 1:1,280 to 1:10,240. Antiserum No. 45 not only reacts with an antigen in human bone tumour cultures which has been demonstrated to be specfiic for C-type virus[11], but

also weakly with the virus isolated from the leukaemic child and passaged in several kinds of cells. A goat antiserum to an endogenous C-type oncornavirus isolated from the BALB/c mouse mammary tumour cell line EMT6 (ref. 12) gives similar reactions. The strongest antiserum to RLV (76), however, does not react with either the human bone tumour cultures or the new virus isolated from the leukaemic child. This finding strongly indicates that the weak but positive reaction with two antisera is not due to contamination of the cultures with a murine virus but to an interspecies antigenic determinant not detected by antiserum No. 76.

A goat antiserum to the purified p27 antigen of the simian sarcoma virus (SSV)[13] gives a good reaction with cultures harbouring the primate virus, although the acetone-fixed slides have been kept for 18 months at −20 °C. The weak reaction with the new virus isolate is probably not only due to poor replication of the virus and in turn to decreased production of p27 in the cytoplasm, but also to an antigenic dissimilarity between the simian and the human virus.

Another source of contamination may be XC cells. Avian Rous sarcoma virus carried in XC cells does not replicate in human cells and is antigenically completely unrelated to mammalian viruses[14]. Control XC cultures proved to be repeatedly negative with all antisera. The cocultivation procedure may have activated an endogenous rat virus. Rat antiserum No. 76, however, reacts with endogenous viruses activated in XC cells by 5-bromodeoxyuridine (20 μg ml⁻¹) and 2% dimethylsulphoxide[15], but not with XC cells cocultivated with the leukaemic cells. Goat antiserum to E6-MuLV shows quite the opposite pattern, indicating that contamination with endogenous rat virus may be excluded.

A second isolation was made 1 month later from the same patient, while in remission, by cocultivating the buffy coat of the peripheral blood with human embryonic fibroblasts FB289. Within 2 weeks virus was demonstrable by electron microscopy, XC plaque test and immunofluorescence. In the latter, rat serum No. 76 again gave no reaction, whereas goat anti-E6-MuLV was positive (1:40). Our conclusion is that we have twice isolated a C-type oncornavirus from a leukaemic child. The virus can replicate in human embryonic cells and in rat XC cells, in which it has a cytopathogenic effect. Possible laboratory contamination with animal oncornaviruses may be excluded. Biochemical studies of this isolate are required to assess in more detail its relationship to animal viruses and to determine whether it is exogenous or endogenous to human cells.

Table 2 Immunofluorescence endpoint titration of cell cultures with antisera to animal C-type oncornaviruses*

Cell lines	Antisera			
	Rat anti-RLV 45	Rat anti-RLV 76	Goat anti-E6-MuLV	Goat anti-SSV1 p27
BALB/c 3T3 control	—	—	—	—
BALB/c 3T3 + RLV	2,560	5,120	2,560	20
XC control	—	—	—	—
XC + leukaemic bone marrow, 2nd passage	40	—	20	—
XC + 5-bromodeoxyuridine + dimethylsulphoxide	160	80	—	40
HEK control	—	—	ND	40
HEK + irradiated XC (+ leukaemic BM)	40	—	ND	ND
HEK + irradiated XC	—	—	ND	ND
FB289	ND	—	ND	ND
FB289 + supernatant infected HEK cells	ND	—	40	—
Woolly monkey fibrosarcoma	320	80	80	20
Human adult fibroblast 1, 17th passage	—	—	—	320
Human chondrosarcoma, 15th passage	80	—	80	10

* Twelve spots on acetone-fixed slides were used for incubation. Nine of the wells were filled with different dilutions of the antiviral serum. Twofold dilutions were made in PBS, beginning at 1:10. One well was used for normal serum, one for only the fluorescein isothiocyanate (FITC)-conjugated anti-IgG serum and one for only PBS.
ND, Not determined. As two different FITC-conjugated antisera to goat IgG reacted with HEK cells the tests with goat antiviral sera were not carried out on HEK cells.

Department, Antoni van Leeuwenhoek Hospital, Amsterdam, and p27 antigen by Dr S. T. August, Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York.

K. NOOTER
A. M. AARSSEN
P. BENTVELZEN

Radiobiological Institute TNO,

F. G. DE GROOT
FRIDA G. VAN PELT

Institute for Experimental Gerontology TNO,
Rijswijk ZH, The Netherlands

1 Tooze, J., *The Molecular Biology of Tumor Viruses* (Cold Spring Harbor, 1974).
2 Hellmann, R., Kufe, D., and Spiegelman, S., *Proc. natn. Acad. Sci. U.S.A.*, 69, 435–439 (1972).
3 Gallo, R. C., Miller, N. R., Saxinger, W. C., and Gillespie, D., *Proc. natn. Acad. Sci. U.S.A.*, 70, 3219–3224 (1973).
4 Todaro, G., and Gallo, R. C., *Nature*, 244, 206–209 (1973).
5 Hirshaut, Y., Reagan, R. L., Perry, S., De Vita, V., Jr, and Barile, M. F., *Cancer*, 34, 1080–1089 (1974).
6 Mak, T. W., Manaster, J., Howatson, A. F., McCulloch, E. A., and Till, J. E., *Proc. natn. Acad. Sci. U.S.A.*, 71, 4336–4340 (1974).
7 Gallagher, R. E., and Gallo, R. C., *Science*, 187, 350–353 (1975).
8 Miller, M. F., Allen, P. T., and Dmochowski, L., *J. gen. Virol.*, 21, 57–68 (1973).
9 Klement, V., Rowe, W. P., Hartley, J. W., and Pugh, W. E., *Proc. natn. Acad. Sci. U.S.A.*, 63, 753–758 (1969).
10 Niwa, O., Declève, A., Lieberman, M., and Kaplan, H. S., *J. Virol.*, 12, 68–73 (1973).
11 Zurcher, C., Brinkhof, J., Bentvelzen, P., and De Man, J. C. H., *Nature*, 254, 457–459 (1975).
12 Rockwell, S. C., Kallman, R. F., and Fajardo, L. F., *J. natn. Cancer Inst.*, 49, 735–750 (1972).
13 Strand, M., and August, J. T., *J. biol. Chem.*, 248, 5627–5633 (1973).
14 Fleissner, E., *et al.*, *Cold Spring Harb. Symp. quant. Biol.*, 39, 1057–1066 (1975).
15 Grollé, P. F., Bentvelzen, P., and Van Noord, M. J., *Biomédicine*, 19, 148–151 (1973).

Fig. 1 Profile of Sepharose 4B agarose filtration of the 38–52% ammonium sulphate fraction of platelet actomyosin before (*A*) and after (*B*) phosphorylation. For both samples, a 1.65 ml aliquot containing 7.5 mg protein was applied to a 1.5 × 88 cm column equilibrated and eluted at 21 ml h^{-1} with 0.5 M KCl 15 mM Tris-HCl (pH 7.5), and 1 mM EDTA and 2.5 mM dithiothreitol. Samples (3.5 ml) were collected of which 0.05 ml was used for ATPase assay (shown above). Ordinate indicates A_{720} of the ATPase assay (control sample was incubated for a longer period of time than the phosphorylated sample). Void volume and salt boundary are indicated (V_0 and $V_0 + V_i$). Fractions were pooled as indicated for SDS–polyacrylamide gel electrophoresis (Fig. 2) and ATPase assays (Table 1). Protein concentration of pooled fractions was 0.2–0.3 mg ml^{-1}.

Phosphorylation of platelet myosin increases actin-activated myosin ATPase activity

HUMAN platelet myosin, which is similar to other myosins isolated from non-muscle cells, has a molecular weight of 460,000 and is composed of two heavy chains (200,000) and two different light chains (20,000 and 15,000)[1-3]. The 20,000 light chain can be phosphorylated by a kinase endogenous to human platelets[3]. This enzyme, which has been isolated and partially purified[4], transfers ^{32}P from γ-^{32}P-ATP to the 20,000 light chain of platelet myosin in the presence of Mg^{2+}. So far, the biological significance of this phosphorylation has been unknown, but we report here that phosphorylation of platelet myosin results in an increase in the actin-activated myosin ATPase activity measured at low ionic strength. Dephosphorylation of phosphorylated myosin results in a decrease in the actin-activated ATPase activity.

A 38–52% ammonium sulphate fraction of platelet actomyosin, containing the platelet myosin light chain kinase, incorporates 0.8–1.0 mol P$_i$ per mol 20,000 light chain[3]. To study the effect of phosphorylation, the unphosphorylated ammonium sulphate fraction was divided in half. One half was phosphorylated with 3 mM MgCl$_2$ and 0.2 mM ATP and incubation at room temperature, pH 7.5 for 30 min. The control half of the sample was treated in the same manner but without ATP or MgCl$_2$. Both the control and the phosphorylated myosin were then chromatographed on columns of Sepharose 4B (Fig. 1). In the case of the phosphorylated sample, agarose filtration terminated phosphorylation.

Two peaks of K$^+$-EDTA-activated ATPase activity were eluted for each sample. As shown in Fig. 2, the first peak of ATPase activity is caused by platelet actomyosin and the second peak by platelet myosin alone. Representative fractions from each peak were analysed for actin-activated myosin ATPase activity measured in the presence of Mg^{2+} at low ionic strength and K$^+$-EDTA- and Ca^{2+}-activated myosin ATPase activity assayed at high ionic strength.

Table 1 shows the effect of phosphorylation on myosin ATPase activity. The low ionic strength Mg^{2+} ATPase activity

of phosphorylated myosin is 0.17 μmol P$_i$ per mg protein per min after addition of rabbit skeletal muscle actin. Control (non-phosphorylated) myosin has a significantly lower specific activity (0.029) after the addition of the same amount of rabbit skeletal muscle actin. K$^+$-EDTA- and Ca^{2+}-activated ATPase activities, measured at high ionic strength, are essentially the same for phosphorylated and control myosin.

The enzymatic activities for the initial peaks (actomyosin) from Sepharose chromatography are also shown in Table 1. Again, the actin-activated ATPase activity for phosphorylated myosin was more than four times greater (0.126 compared with 0.028) than for non-phosphorylated myosin. The ATPase activity for phosphorylated myosin without added actin (0.052) is increased, reflecting the presence of platelet actin (Fig. 2).

Having found an increase in the actin-activated myosin ATPase activity after phosphorylation, it was interesting to see if dephosphorylation would decrease the enzymatic activity. Myosin was first phosphorylated with γ-^{32}P-ATP to contain 0.8–1.0 mol phosphate per mol 20,000 light chain[3]. Labelled myosin was incubated with *Escherichia coli* alkaline phosphatase which released 70–80% of the covalently bound ^{32}P in 10 h, whereas a control sample incubated in the absence of alkaline phosphatase retained all incorporated ^{32}P.

Both dephosphorylated myosin and the control sample were chromatographed on a column of Sepharose 4B (for the
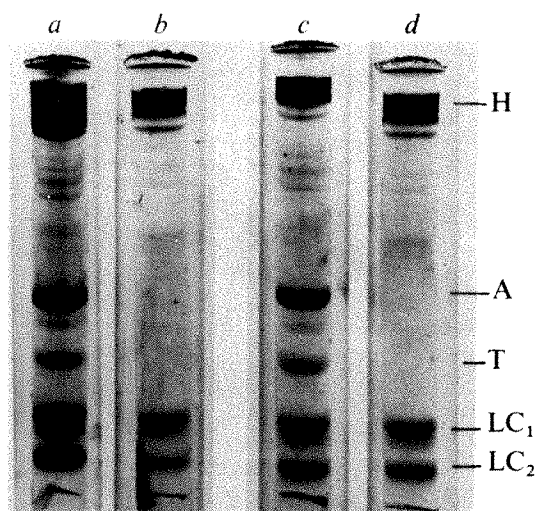
Fig. 2 1% SDS–7.5% polyacrylamide gels of non-phosphorylated (*a, b*) and phosphorylated (*c, d*) platelet actomyosin (*a, c*) and myosin (*b, d*) eluted from Sepharose 4B (Fig. 1). Letter above each gel refers to the pooled fraction indicated on the Sepharose 4B elution profile in Fig. 1. H, myosin heavy chain; A, actin; T, tropomyosin; LC₁, 20,000 myosin light chain; LC₂, 15,000 myosin light chain. Dye marker can be seen below LC₂. Migration was from top to bottom. Gel electrophoresis was carried out as described previously[10].

Table 2 Effect of dephosphorylation on ATPase activity

| | ATPase (μmol P_i per mg protein per min) | | | |
| | Actin-activated | | K^+-EDTA | Ca^{2+} |
	$-$Actin	$+$Actin		
Phosphorylated myosin	0.037	0.14	0.53	0.55
Dephosphorylated myosin	0.013	0.04	0.40	0.40

Labelling of platelet myosin: conditions for phosphorylation were as in Table 1 except that γ-^{32}P-ATP was premixed with ATP to give a final concentration of 20 μCi ml⁻¹. Labelled samples were dialysed against 0.5 M KCl, 15 mM Tris-HCl (pH 7.5) and 2.5 mM dithiothreitol before dephosphorylation.

Conditions for dephosphorylation: 2.8 ml labelled myosin (35–55% ammonium sulphate fraction, 9 mg ml⁻¹, 600,000 c.p.m. mg⁻¹) was adjusted to pH 8.0 with Tris base and divided into two equal aliquots. To one, alkaline phosphatase (*E. coli*; BAPF, Worthington) was added in a ratio 1:7 (mg/mg, enzyme/substrate). To the other, an equivalent volume of 65% ammonium sulphate was added. Both samples were incubated at 23 °C for 10–22 h. Incubation was terminated by Sepharose 4B chromatography of the entire sample (1.5 ml) using the same size column and elution buffer detailed in Fig. 1. Concentration of the pooled fraction eluted from the column was 0.3–0.4 mg ml⁻¹. ^{32}P release was measured by precipitating aliquots (50–100 μg) in cold 10% Cl₃CCOOH, 2% sodium pyrophosphate, heating at 96 °C for 20 min, chilling on ice and filtering the precipitate through Millipore HA filters in a Millipore sampling manifold. Filters were washed repeatedly with cold 5% Cl₃CCOOH, 1% sodium pyrophosphate and counted in Aquasol (New England Nuclear) in a Packard Tri-Carb liquid scintillation counter. Identification of 20,000 myosin light chain as the only phosphorylated protein has been described previously[3]. Conditions for ATPase assay are given in Table 1. Data are from duplicate determinations on three different preparations.

sample treated with alkaline phosphatase, this was necessary to separate the enzyme from myosin). Tubes containing myosin ATPase activity were pooled and assayed for actin activation. Table 2 shows the effect of dephosphorylation on actin activation: a decrease in actin-activated ATPase activity from 0.14 to 0.04 μmol P_i per mg protein per min. The K⁺-EDTA- and Ca²⁺-activated myosin ATPase activities measured at high ionic strength were not significantly altered. Furthermore, when the two samples were electrophoresed in 1% SDS-7.5%

Table 1 Effect of phosphorylation on ATPase activity

| | | ATPase (μmol P_i per mg protein per min) | | | |
| | | Actin-activated* | | K^+-EDTA | Ca^{2+}† |
		$-$Actin	$+$Actin		
Myosin	Phosphorylated	0.006	0.170	1.00	0.41
	Control	0.002	0.029	0.90	0.38
Actomyosin	Phosphorylated	0.052	0.126	0.54	0.37
	Control	0.007	0.028	0.38	0.38

Conditions for phosphorylation: 1.5 ml 38–52% ammonium sulphate fraction of platelet actomyosin[1] (3.5–6 mg ml⁻¹) was incubated in 0.1 M KCl, 5 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4 mM MgCl₂, 0.2 mM ATP and 2.5 mM dithiothreitol at 23 °C for 30 min (ref. 3) (total volume 1.65 ml). Phosphorylation was terminated by chromatography of the entire sample on Sepharose 4B. Control samples were treated in a like manner with the omission of MgCl₂ and ATP.

* Assay conditions: 10 mM Tris-HCl (pH 7.2), 1 mM ATP, 1.4 mM MgCl₂ and 30 mM KCl. Platelet myosin or actomyosin 0.06–0.10 mg ml⁻¹, rabbit skeletal muscle actin, 0.6–1.0 mg ml⁻¹, 37 °C.

Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt[7] and migrated as a single band in 1% SDS-7.5% polyacrylamide gel electrophoresis and 8 M urea-polyacrylamide gel electrophoresis, pH 8.6. Specific activity excludes added rabbit skeletal actin.

† Assay conditions: 20 mM Tris-HCl (pH 7.2), 2 mM ATP, 2 mM EDTA or 10 mM CaCl₂ and 0.5 M KCl. Platelet myosin or actomyosin, 0.02–0.04 mg ml⁻¹, 37 °C.

Data are from duplicate determination on three different preparations. P_i production was measured by a modification of the method of Martin and Doty[8]. Phosphate production was linear with time during the period used for assay (0–60 min). Protein concentration was determined by the method of Lowry *et al.*[9] after Cl₃CCOOH precipitation, bovine serum albumin was used as standard.

polyacrylamide gels, the patterns were identical. Gel electrophoresis revealed the presence of platelet actin in these preparations, similar to Fig. 2*a* and *c*, and this is reflected in the ATPase activities (Table 2).

Phosphorylation of a light chain from skeletal and cardiac muscle myosin by a kinase isolated from rabbit sarcoplasm has been reported[5]. This kinase differs from that purified from platelets by us in requiring Ca²⁺ for activity[4]. In addition, the platelet myosin light chain kinase does not phosphorylate skeletal or cardiac muscle myosin but does phosphorylate mouse fibroblast[6] and chicken gizzard myosin[4].

The identification of actin and myosin in a large number of vertebrate as well as invertebrate non-muscle cells[2] raises questions as to how the interaction of these proteins is controlled. We have described the effect of phosphorylating the 20,000 light chain of a non-muscle myosin: an increase in the actin-activated myosin ATPase activity. The finding that platelet myosin light chain kinase phosphorylates the 20,000 light chain of fibroblast and smooth muscle myosin suggests that phosphorylation may have a role in controlling actin–myosin interaction in other cells.

ROBERT S. ADELSTEIN
MARY ANNE CONTI

Section on Molecular Cardiology,
Cardiology Branch,
National Heart and Lung Institute,
Bethesda, Maryland 20014

1 Adelstein, R. S., and Conti, M. A., *Cold Spring Harb. Symp. quant. Biol.*, 37, 599–606 (1972).
2 Pollard, T. D., and Weihing, R. R., *Critical Reviews in Biochemistry*, 2, 1–65 (CRC, Cleveland, Ohio, 1974).
3 Adelstein, R. S., Conti, M. A., and Anderson, W., Jr, *Proc. natn. Acad. Sci. U.S.A.*, 70, 3115–3119 (1973).
4 Adelstein, R. S., Daniel, J. L., Conti, M. A., and Anderson, W., Jr, *Proc. Fed. Eur. Biochem. Soc., Ninth FEBS Meeting, Budapest* (in the press).
5 Pires, E., Perry, S. V., and Thomas, M. A., *FEBS Lett.*, 41, 292–296 (1974).
6 Conti, M. A., and Adelstein, R. S., *Fedn Proc.*, 34, 670a (1975).
7 Spudich, J. A., and Watt, S., *J. biol. Chem.*, 246, 4866–4871 (1971).
8 Martin, J. B., and Doty, D. M., *Analyt. Chem.*, 21, 965–967 (1949).
9 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. biol. Chem.*, 193, 265–275 (1951).
10 Fairbanks, G., Steck, T. L., and Wallach, D. F. H., *Biochemistry*, 10, 2606–2617 (1971).

Oxygen reduction and optimum production of ATP in photosynthesis

THE accepted pathway of CO_2 fixation in plant photosynthesis requires that the photosynthetic light reactions produce ATP and reduced pyridine nucleotide (NADPH) in the molar ratio 3:2 (ref. 1). Early studies of photosynthetic phosphorylation suggested that non-cyclic electron transport could produce only equimolar amounts of ATP and NADPH, and the source of the extra ATP was presumed to be cyclic electron flow[2]. The view that the non-cyclic system is by itself able to produce twice as much ATP as NADPH has been expressed[3,4], and removes the need for the *in vivo* operation of a cyclic electron flow which can be demonstrated *in vitro* only in artificial conditions[5]. An inflexible ATP-NADPH ratio of 2:1 for the products of the light reactions would, however, result in a feedback inhibition of electron transport, with ADP concentration as the limiting factor. One way of achieving flexibility in the relative production of ATP and NADPH would be for a low
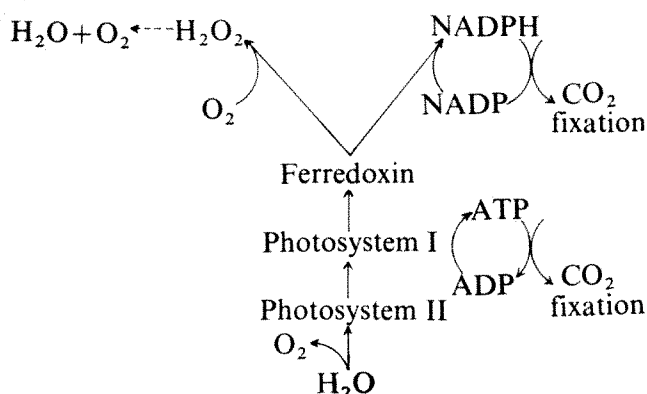
Fig. 1 The combined reduction of oxygen and of NADP by non-cyclic electron transport. The reaction of ferredoxin with oxygen makes possible the synthesis of ATP without the production of NADPH. Stoichiometries are discussed in the text and are not implied in the diagram.

absolute stoichiometry of non-cyclic photophosphorylation (corresponding perhaps to a 1 : 1 ratio) to be accompanied by an ancillary reaction producing ATP but not NADPH. Such an ancillary reaction would be especially important in situations where ATP might be required for phosphorylations additional to those of the reductive pentose phosphate pathway.

Figure 1 outlines a mechanism by which an optimum balance
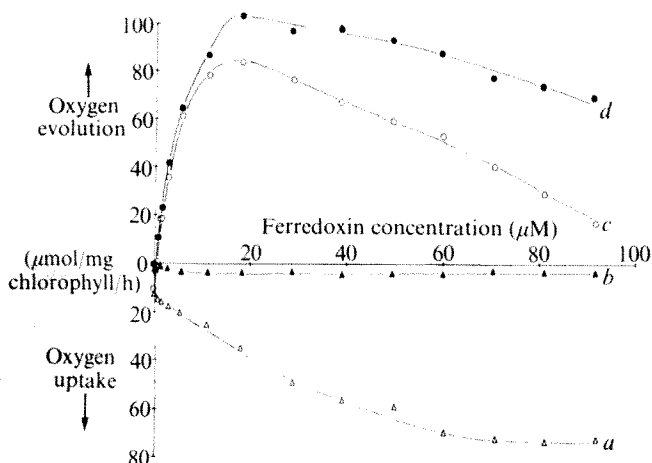
of ATP and NADPH could be maintained by non-cyclic electron transport in photosynthesis. Here the ancillary reaction is assumed to be oxygen-reducing electron transport, and the mechanism, therefore, relies on a "pseudocyclic" contribution to overall ATP synthesis, as suggested previously by Heber[6]. The novel feature of this scheme is a competition for reduced ferredoxin by NADP and oxygen. Production of too little ATP would inhibit the dark regeneration of NADP, and ferredoxin would then be oxidised only by oxygen. With oxygen as the effective electron acceptor, ATP but not NADPH would be produced, and a higher concentration of ATP would be restored. Excess ATP would lead to pyridine nucleotide being present predominantly in the oxidised form, and thus to electrons being diverted from oxygen to NADP. In this way the active non-cyclic electron transport chain would continually meet the requirements of CO_2 fixation, and would be able to respond to any changes in the metabolic demands made on it by synthesis of carbohydrate, protein or lipid.

Ferredoxin is an effective mediator of photosynthetic oxygen uptake by isolated chloroplasts[7], a reaction which accompanies non-cyclic (in this case also termed "pseudocyclic") photophosphorylation[8]. It is also the penultimate component of the photosynthetic electron transport chain, and as such is essential for the reduction of pyridine nucleotide in photosynthesis[9]. The hypothesis represented in Fig. 1 requires ferredoxin to perform both of these functions simultaneously, a situation which is demonstrated experimentally in Fig. 2. In the absence of both NADP and catalase, the rate of photosynthetic oxygen uptake by isolated chloroplasts is a function of ferredoxin concentration (curve *a*), and this oxygen uptake is abolished by addition of catalase (curve *b*). The photosynthetic oxygen-consuming reaction can, therefore, be written as the reversed dismutation of hydrogen peroxide:

$$H_2O + \tfrac{1}{2}O_2 \rightarrow H_2O_2$$

This describes non-cyclic transfer of one electron pair from water to oxygen. In the presence of NADP a catalase-insensitive evolution of oxygen should occur as transfer of one electron pair from water to NADP results in the reaction:

$$H_2O + NADP^+ \rightarrow NADPH + H^+ + \tfrac{1}{2}O_2$$

In this situation, however, catalase stimulates observed oxygen evolution (curves *c* and *d*), and the degree of this stimulation increases with increasing ferredoxin concentration. This stimulatory effect of catalase on net oxygen evolution can be considered to be no more than an inhibitory effect of catalase on a "background" oxygen uptake. If, of each pair of electrons from water, an average of *n* electrons are transferred to oxygen and $2 - n$ to NADP, then the overall reaction occurring in the

Fig. 2 The effects of catalase on ferredoxin-mediated uptake and overall evolution of oxygen by illuminated spinach chloroplasts. For curves *c* and *d* oxygen evolution was supported by the presence of NADP (2 mM). Curves *b* and *d* represent rates obtained in the presence of 8×10^3 U catalase (Boehringer). For curves *a* and *c* sodium azide (2 mM) was present as an inhibitor of any endogenous catalase.

Oxygen exchange was measured in a Rank oxygen electrode, with illumination by two 300-W slide projectors. Broken, washed chloroplasts had been isolated from spinach by a method described previously[14], and the reaction vessel contained sorbitol (0.1 M), $MgCl_2$ (5 mM), NaCl (20 mM), EDTA (2 mM), HEPES (pH 7.5, 50 mM), NH_4Cl (5 mM) and chloroplasts (100 μg of chlorophyll) in a final volume of 2 ml. Ferredoxin, isolated from *Spirulina maxima* by the method of Hall *et al.*[15], was added as a 1.4-mM solution. Substantially similar results have been obtained with a number of plant-type ferredoxins (unpublished work). The extent of stimulation of net oxygen evolution by catalase is apparently unaffected both by omission of NH_4Cl and by addition (in the absence of NH_4Cl) of ATP or ADP with phosphate (these results also unpublished).

absence of catalase activity (curve *c*) becomes

$$H_2O + \frac{n}{4}O_2 + [(2-n)/2]NADP^+ \rightarrow \frac{n}{2}H_2O_2 +$$

$$+ [(2-n)/2](NADPH + H^+) + [(2-n)/4]O_2$$
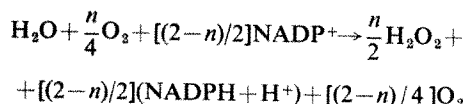
and the ratio of oxygen molecules evolved to electron pairs transferred is given by

$$(O_2/2e^-)_{-cat} = [(2-n)/4] - (n/4) = [(1-n)/2]$$

In the presence of catalase, however, the compensatory reaction

$$\frac{n}{2}H_2O_2 \rightarrow \frac{n}{4}H_2O + \frac{n}{4}O_2$$
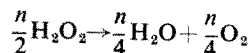
also occurs (curve *d*), and so the corresponding ratio is merely

$$(O_2/2e^-)_{+cat} = [(2-n)/4]$$

For any absolute rate of electron transport which is unaffected by catalase, the ratio *r* of the observed rates of net oxygen evolution in the presence (v_{+cat}) and absence (v_{-cat}) of catalase will be given by

$$r = v_{+cat}/v_{-cat} = (O_2/2e^-)_{+cat}/(O_2/2e^-)_{-cat}$$
$$= (2-n)/[2(1-n)]$$

and thus *n* ($0 \leqslant n \leqslant 2$) can be calculated from the relationship

$$n = [1/(1-2r)] + 1$$

In Fig. 3*a* this measure of the contribution of the oxygen-reducing reaction to total electron flux has been calculated
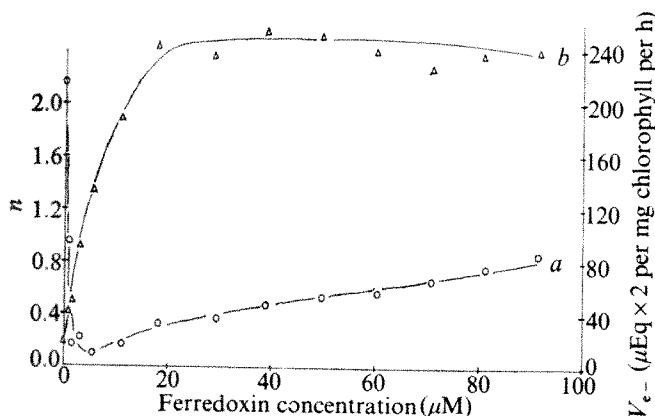
Fig. 3 *a*, *n* is the average number of electrons which go to oxygen, per pair transferred through the photosynthetic chain. *b*, v_e- is the rate of electron transport in μEq ×2 per mg chlorophyll per h, units comparable with similar units involving μatoms oxygen exchanged or μmol NADP reduced. Values for each variable (*n* and v_e-) were calculated as described in the text from the data in Fig. 2, with individual values corresponding to observed rates of oxygen evolution at each ferredoxin concentration.

from the data in Fig. 2, and is plotted against the same scale of ferredoxin concentration. As v_{-cat} is related to the absolute rate of electron transport (v_{e-}) as follows

$$v_{-cat}/v_{e-} = (1-n)/2$$

the absolute rate of electron flux can also be calculated. The dependence of electron transport *per se* on ferredoxin concentration is shown in Fig. 3*b*. As in the case of net oxygen evolution (Fig. 2), a saturated rate of electron transport is achieved at a ferredoxin concentration of 15–20 μM. Unlike net oxygen

evolution, however, the rate of electron transport does not significantly decrease with higher ferredoxin concentrations. The decline in the observed rate of oxygen evolution with high ferredoxin concentrations (Fig. 2) presumably reflects an increasing displacement of NADP by oxygen as the terminal electron acceptor (Fig. 3*a*). At these saturating ferredoxin concentrations of >15 μM n ⩾0.3, and so not less than 15% of total electron transport is supported by reduction of oxygen.

These results question Arnon's statement[8] that in photosynthesis reduction of oxygen cannot occur simultaneously with reduction of NADP. Using mass spectrometry Egneus *et al.*[10] have identified an uptake of $^{18}O_2$ by CO_2-fixing chloroplasts in a reaction apparently unrelated to glycolate synthesis. This provides independent support for a competition of oxygen with NADP for electrons from the photosynthetic chain. Whitehouse *et al.*[11] have suggested that a significant rate of reduction of oxygen occurs in the presence of added FMN even where the artificial electron acceptor ferricyanide is used instead of NADP and ferredoxin.

The importance to photosynthesis of electron flow to oxygen has also been suggested by Heber, whose detailed measurements of the quantum requirements of various photoreductions support a contribution of oxygen-reducing electron transport to overall ATP synthesis[6], and point to a flexibility in the stoichiometry of photosynthetic phosphorylation and NADP reduction[12]. The work of Patterson and Myers[13] with *Anacystis nidulans* indicates that an *in vivo* photosynthetic production of hydrogen peroxide may indeed occur.

I thank the SRC for a research studentship. The ferredoxin was a gift from Dr K. K. Rao.

J. F. Allen*

University of London, King's College,
Department of Plant Sciences,
68, Half Moon Lane,
London SE24 9JF, UK

1 Gregory, R. P. F., *Biochemistry of Photosynthesis*, (Wiley, London, 1971).
2 Arnon, D. I., Whatley, F. R., and Allen, M. B., *Science*, 127, 1026-1034 (1958).
3 West, K. R., and Wiskich, J. T., *Biochim. biophys. Acta*, 292, 197-205 (1973).
4 Reeves, S. G., and Hall, D. O., *Biochim. biophys. Acta*, 314, 66-78 (1973).
5 Simonis, W., and Urbach, W., *A. Rev. Pl. Physiol.*, 24, 89-114 (1973).
6 Heber, U., *Biochim. biophys. Acta*, 305, 140-152 (1973).
7 Telfer, A., Cammack, R., and Evans, M. C. W., *FEBS Lett.*, 10, 21-24 (1970).
8 Arnon, D. I., Tsujimoto, H. Y., and McSwain, B. D., *Nature*, 214, 562-566 (1967).
9 Buchanan, B. B., and Arnon, D. I., *Adv. Enzymol.*, 33, 119-176 (1970).
10 Egneus, H., Heber, U., and Matthieson, U., *Hoppe Seylers Z. physiol. Chem.*, 355, 1190 (1974).
11 Whitehouse, D. G., Ludwig, L. J., and Walker, D. G., *J. exp. Bot.*, 73, 772-791 (1971).
12 Heber, U., and Kirk, M. R., *Biochim. biophys. Acta*, 376, 136-150 (1975).
13 Patterson, C. O. P., and Myers, J., *Pl. Physiol.*, Lancaster, 51, 104-109 (1973).
14 Allen, J. F., and Hall, D. O., *Biochem. biophys. Res. Commun.*, 52, 856-862 (1973).
15 Hall, D. O., Rao, K. K., and Cammack, R., *Biochem. biophys. Res. Commun.*, 47, 798-802 (1972).

Agonism and antagonism of γ-aminobutyric acid

EVIDENCE that (+)bicuculline (BIC) is a competitive antagonist of the central inhibitory transmitter γ-aminobutyric acid (GABA) is based[1] mainly on apparent similarities between the molecular structures of BIC, GABA and agonists of GABA. Until recently, there has not been any explanation of the differing potencies of the agonists, or why BIC is an antagonist instead.

In 1970 it was noted[1] that the N and lactone C–C=O in BIC could be isosteric with the N and carboxylate COO of an extended (that is fully *trans*) GABA molecule (zwitterion at physiological *p*H) and its equivalent (O–C=N) in the agonist muscimol. Alternatively[2], a partially folded GABA molecule could have its N and COO congruent with the N and lactone

O–C=O of BIC. The discovery of conformationally restricted GABA agonists[3,4], notably 4-aminotetrolic acid[3], however, gave some support to the extended charge separation—measurements[3] on Drieding models gave 5.2–5.8 Å. General agreement was also obtained from EHT MO predictions of minimum-energy conformations for isolated molecules of GABA[5], muscimol[5] and other agonists[6,7], and for protonated BIC (HBIC—as administered)[6]: a value of 5.8 ± 0.2 Å was obtained[7] if intercharge distances were related to the onium → one carboxylate O in the GABA zwitterion and the onium →

suggests that there is more to its "recognition" of a drug molecule than a single charge separation.

The feature of structure not included in any of the discussions, but now available from CNDO/2 calculations (refs. 9, 22 and unpublished work), is the distribution of charge within the molecules. (Such details were used in the conformation against potency correlation[12] above.) Comparisons of the three-dimensional patterns of charge show features that may distinguish between agonists and antagonists. The charge distribution in the lactone ring in HBIC is of particular interest (Fig. 1a),
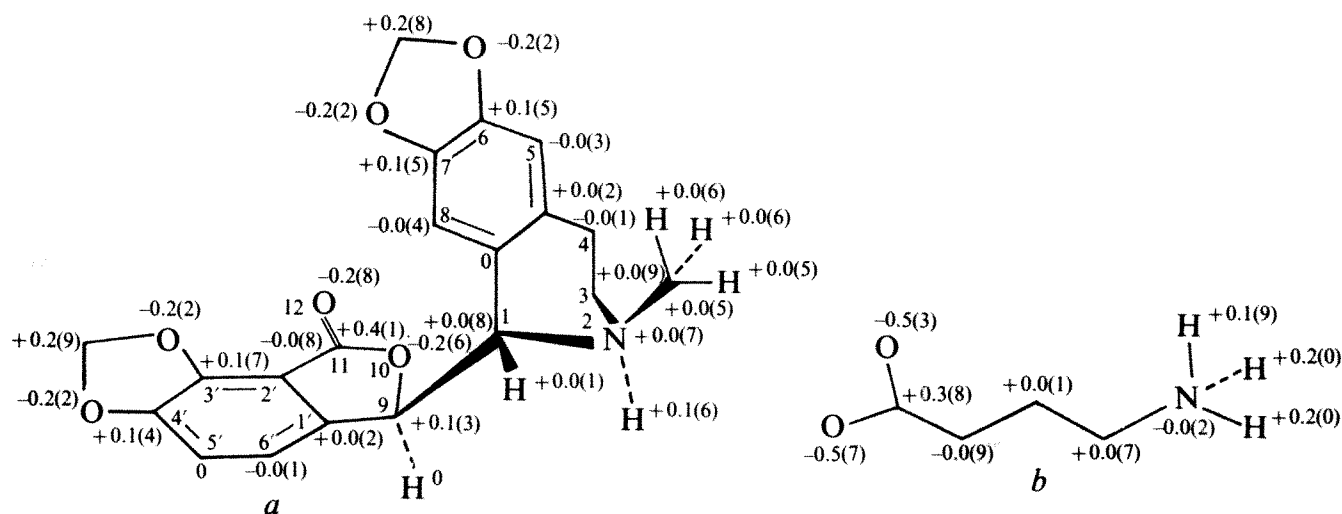
Fig. 1 Skeletal outlines and associated charges (a.u.) for: *a*, HBIC in approximate solution-conformation $(T \sim -90°)$; *b*, GABA zwitterion (charges not significantly altered when molecule is partially folded as described in text). Torsion angle T(N2.C1–C9.O10), positive when N2.C1 is rotated clockwise to eclipse C9.O10, as viewed along C1→C9. (H atoms not shown have a charge of 0.0(5) or less.)

carbonyl O in BIC. Although the EHT method is inadequate with strongly polar molecules[8], and the more folded conformations that would be expected in flexible molecules are predicted by CNDO/2 (ref. 9) and PCILO[10,11] MO calculations, general agreement with EHT results is fortuitously re-established when allowance is made in the CNDO/2 calculations for an aqueous medium[9].

A preliminary analysis of the role of agonist flexibility has now revealed[12] a correlation between relative potency and interaction probability calculated from (potential energy) conformation population distributions corrected for the charge-separation tolerance of the receptor.

Concerning BIC as a competitive antagonist, the crystal structure determination[13,14] of (+)BIC showed[15,16] that the model originally portrayed[1] was the wrong diastereomer—adlumidine (1S,9S)[17] instead of 1S,9R (ref. 17) (Fig. 1a). Also the methyl substituent at the N is pseudoaxial to the pyridine ring, not pseudoequatorial. Theoretical calculations show, however, that in HBIC[6,13,18], and in the potent N-MeBIC[13,18], there is appreciable torsional freedom about C1–C9 from about $-60°$ to $-120°$, and within this angular range it is again possible to obtain the isosteric match with GABA which was originally reported[1] for the wrong BIC configuration. Congruence with a partially folded GABA[2] is not easily attainable but it could be achieved accurately by δ-aminovaleric acid, a GABA agonist[19]. The BIC torsion-angle range also makes available a flexibility of charge separation comparable with that of some GABA agonists. BIC should therefore be "recognised" by the GABA receptor as an agonist[20], and its action as a competitive antagonist, by which it blocks the permeability change in the postsynaptic membrane, may therefore be due to some further feature of its structure. The report[21] that (–)BIC is inactive is relevant. Although such selectivity is common, this evidence that the GABA receptor is chirally specific

showing an appreciable difference between the alternatives described earlier, C2′.C11.O12 and O10.C11.O12; the second bears a closer resemblance to the COO of an amino acid zwitterion. More significant, however, is the smaller charge on the above negatively charged atoms in the lactone ring compared with those in the GABA COO group (Fig. 1b). This, and the differences in the spread of positive charge, are the first intimations of a possible mechanism of competitive antagonism: a charge pattern sufficiently similar to that of an agonist to be "recognised" and attracted to a receptor, but with charges too weak and/or diffuse, or too shielded by the remainder of the molecule, to enable the receptor to bind the drug and then undergo the conformational change believed to be associated with the final stages in the action of a transmitter. The charges on the remainder of the BIC molecule may have a role, perhaps contributing to binding around the receptor site, preventing fast removal of the antagonist molecule and thus blocking the receptor.

Further analysis of the causes of the chiral selectivity of BIC and the mechanism of its antagonism will be aided by results of physiological tests on the other related phthalideisoquinoline alkaloids.

E. G. STEWARD
P. W. BORTHWICK
G. R. CLARKE
D. WARNER

Department of Physics,
The City University,
London EC1V 4PB, UK

[1] Curtis, D. R., Duggan, A. W., Felix, D., and Johnston, G. A. R., *Nature*, **226**, 1222 (1970).

2 Steward, E. G., Player, R. B., Quilliam, J. P., Brown, D. A., and Pringle, M. J., *Nature new Biol.*, 233, 87 (1971).
3 Beart, P. M., Curtis, D. R., and Johnston, G. A. R., *Nature new Biol.*, 234, 80 (1971).
4 Johnston, G. A. R., *et al.*, *J. Neurochem.*, 24, 157 (1975).
5 Kier, L. B., and Truitt, E. B., *Experientia*, 26, 988 (1970).
6 Kier, L. B., and George, J. M., *Experientia*, 29, 501 (1973).
7 Kier, L. B., George, J. M., and Höltje, H.-D., *J. Pharmaceut. Sci.*, 63, 1435 (1974).
8 Kier, L. B., *Molecular Orbital Theory in Drug Research* (Academic, New York, 1971).
9 Warner, D., and Steward, E. G., *J. molec. Struct.*, 25, 403 (1975).
10 Pullman, B., and Berthod, H., *C. r. hebd. Séanc. Acad. Sci., Paris*, 278, 1433 (1974).
11 Pullman, B., and Berthod, H., *Theor. chim. Acta*, 36, 317 (1975).
12 Steward, E. G., and Clarke, G. R., *J. theoret. Biol.* (in the press).
13 Gilardi, R. D., *Nature new Biol.*, 245, 86 (1973).
14 Gorinsky, C., and Moss, D. S., *J. Cryst. molec. Struct.*, 3, 299 (1973).
15 Steward, E. G., Player, R. B., and Warner, D., *Nature new Biol.*, 245, 88 (1973).
16 Johnston, G. A. R., and Curtis, D. R., *Nature*, 248, 804 (1974).
17 Snatzke, G., *et al.*, *Tetrahedron*, 25, 5059 (1969).
18 Andrews, P. R., and Johnston, G. A. R., *Nature new Biol.*, 243, 29 (1973).
19 Swagel, M. W., Ikeda, K., and Roberts, E., *Nature new Biol.*, 246, 91 (1973).
20 Warner, D., Player, R. B., and Steward, E. G., *Int. Union of Crystallography First European Meeting*, B4, (1973).
21 Collins, J. F., and Hill, R. G., *Nature*, 249, 845 (1974).
22 Warner, D., Borthwick, P. W., and Steward, E. G., *J. molec. Struct.*, 25, 397 (1975).

Differences in natural carbon isotope ratios of milk and hair from cattle grazing tropical and temperate pastures

ABOUT 99% of all carbon is the ^{12}C isotope while 1% is ^{13}C. The precise ratio of the isotopes will vary depending on the material analysed. In plants, fractionation of carbon is brought

reported for temperate and tropical pastures (ref. 3 and M.M.L., J.H.T., and R.J.J., unpublished). There were also large differences between the ratios for milk and hair produced from C_3 and C_4 pastures, although the mean difference was reduced from -14.0% with pasture to -9.8% for the tissues. This narrowing of the ratio may be due to differences in discrimination against ^{13}C during transformations by the rumen microorganisms or within the tissues of the animal. Alternatively, the animals grazing C_4 pastures may have selectively grazed a small amount of C_3 weeds which would increase the carbon ratio relative to that of the feed sample. Similarly, selection of C_4 weeds in C_3 pastures would reduce the ratio below that expected. Values for milk and hair were identical at three of the four locations, indicating that differences in the carbon ratios of the feeds are reflected in both long term and short term animal products. The cause of the difference in ratios for milk and hair at Swan's Lagoon is unknown. Since the carbon ratio of milk and hair are similar, it is likely that the ratios for other animal tissues, including muscle and fat, will show similar differences between feeds.

These results show that the carbon ratios of animals reflect the carbon ratio of the feed being eaten, and that these ratios can be used as a naturally occurring marker of carbon. This finding could have important implication for animal physiology, forensic science and studies of animal evolution. It should be possible to calculate the proportion of carbon in milk coming from feed and body reserves or to determine the

Table 1 $^{13}C/^{12}C$ ratios of pasture and of milk and hair of grazing cattle

Pasture location		Pasture species	Photosynthetic pathway	No. of cows	Time since calving (d)	$\delta^{13}C$ parts per ml		
						Pasture	Milk	Hair
Swan's Lagoon, Qld	20°10'S, 147°15'E	*Heteropogon contortus*	C_4	3	45	-14.0	-15.5	-12.1
Wollongbar, NSW	28°50'S, 153°25'E	*Pennisetum clandestinum*	C_4	3	107	-12.4	-15.0	-15.1
Murray Bridge, SA	35°07'S, 139°16'E	*Lolium perenne	C_3	1	120	-25.4	-22.5	-22.3
Werribee, Vic.	37°54'S, 144°39'E	*Lolium perenne	C_3	1	210	-28.9	-26.0	-26.2

*Also *Trifolium repens* and other temperate species.

about primarily by carbon dioxide assimilation in photosynthesis and is due to preferential utilisation of ^{12}C and exclusion of ^{13}C. Curiously enough, it has been found recently[1,2] that higher plants which fix carbon dioxide by way of the Calvin C_3 cycle pathway differ in $^{13}C/^{12}C$ ratios from plants which fix carbon dioxide through the C_4-dicarboxylic acid pathway. Temperate pasture species fix carbon by way of the Calvin pathway and have $^{13}C/^{12}C$ ratios of approximately -28% (ref. 3 and M.M.L., J.H.T., and R. J. Jones, unpublished), whereas tropical pasture grasses fix carbon through the C_4-dicarboxylic acid pathway[4] and have $^{13}C/^{12}C$ ratios of approximately -12% (ref. 2 and M.M.L., J.H.T., and R.J.J., unpublished). $^{13}C/^{12}C$ ratios are expressed relative to a carbonate standard[5].

Smith and Epstein[5] suggested that isotope ratios of marine animal tissues reflect the ratio in their presumed diet, but there seem to have been no direct comparisons of the isotope ratios of higher animal tissue with those of the food eaten. Our aim, therefore, was to compare the carbon ratios of tissues from animals grazing pasture with contrasting $^{13}C/^{12}C$ ratios. The tissues sampled were hair and milk, representing the products of long and short term absorption of nutrients respectively, whereas the pastures grazed were composed of either temperate C_3 species or tropical C_4 grasses, each grazed at two locations (Table 1). Pasture was the only source of carbon except at Wollongbar where 2–3% of the predominantly C_4 diet consisted of bran/oaten chaff (C_3). The isotope measurements of pasture, hair and milk samples were made with a ratio mass spectrometer[6]. Differences between single replicates are usually less than 1‰.

The carbon ratios of the pasture samples (Table 1) reflected their botanical composition with values close to those previously

rate of exchange of body protein and fat. This would be achieved by feeding a completely C_4 diet to animals that had previously been given only C_3 feeds so that all their tissues had carbon ratios characteristic of C_3 species. In temperate zones C_4 diet could be based upon either the forage or grain of *Zea mays* or *Sorghum bicolor*. Alternatively, animals on a C_4 diet could be changed to one comprised of C_3 feeds. In forensic science this observation could prove valuable in determining the diet and hence the origin of animals or animal products. It should also be possible to determine the diets of ancient animals by measuring the carbon isotope ratios of their remains.

We thank Ms J. Clark, Dr J. C. Radcliffe and Messrs R. G. Holroyd and A. J. E. Royal for providing the samples of pasture, milk and hair.

DENNIS J. MINSON
MERVYN M. LUDLOW

Division of Tropical Agronomy, CSIRO,
Cunningham Laboratory, St Lucia,
Brisbane 4067, Australia

JOHN H. TROUGHTON

Physics and Engineering Laboratory, DSIR,
Lower Hutt, New Zealand

1 Bender, M. M., *Radiocarbon*, 10, 468–72 (1968).
2 Tregunna, E. B., Smith, B. N., Berry, J. A., and Downton, W. J. S., *Can. J. Bot.*, 48, 1209–1214 (1970).
3 Smith, B. N., and Brown, W. V., *Am. J. Bot.*, 60, 505–513 (1973).
4 Ludlow, M. M., and Wilson, G. L., *Aust. J. biol. Sci.*, 24, 449–470 (1971).
5 Smith, B. N., and Epstein, S., *Pl. Physiol., Lancaster*, 46, 738–742 (1970).
6 Troughton, J. H., in *Photosynthesis and Photorespiration* (edit. by Hatch, M. D., Osmond, C. B., and Slatyer, R. O.), 124–129 (Wiley Interscience, New York, 1971).

reviews

Makings of a great physiologist

THIS book* is largely directed to a critical account of the development of the ideas and of the experimental research of the great French physiologist, Claude Bernard, during the period 1842–48. From 1841–44 Bernard was *preparateur* to Magendie at the Collège de France but he was already slowly evolving into the great creative investigator that he later became, with ideas and discoveries that ultimately overshadowed those of his teacher. In his discussion of the research Bernard carried out between 1842 and 1848 Professor Holmes has focused on studies of digestion, nutrition and related problems. Although the subject of the study is Claude Bernard much of the book is necessarily concerned with the activities of others, particularly with the adventures in animal chemistry of the two great organic chemists Liebig and Dumas, with their respective supporters.

In his thesis for the Doctorate of Medicine published in 1843 under the title *Du suc gastrique et de son rôle dans la nutrition*, Bernard described the results of investigations which revealed that when sucrose is injected into the veins of a dog it is excreted in the urine largely or completely unchanged; but sucrose given preliminary treatment with digestive juices before its intravenous injection is not so excreted. This was a very important discovery for a young man but afterwards, until 1848, Bernard published few scientific articles.

For a study of Bernard's early scientific development Professor Holmes has been able to consult much manuscript material from the Collège de France, including Bernard's early laboratory notebooks, classified and catalogued between 1962 and 1965 by Professor M. D. Grmek. As Professor Holmes writes in the introduction to his book, "Bernard's notebooks show that the papers he published in this period [1843–48] were only superficial markers of the trail that he was following. . . . His reasoning was often incisive, but occasionally vague, just as his experimental procedures were in some respects unusually rigorous but in

"For more than two years, at the beginning of my career, I wasted my time pursuing theories and chimeras. It was not until after a long period of deception that I ended by reflecting and thinking that . . . my will could not change the laws of nature".

CLAUDE BERNARD

Ronan Picture Library

other respects below the best standards of his time". Professor Holmes's book throws important light on the years of preparation which until now have been very little documented. We can now see for ourselves the evidence for what I suppose most of us guessed at all along, that Bernard must have gone through periods of doubt and made false starts, had prejudices and frustrations, and that in fact he did not spring fully armed into the scientific arena but went through the painful processes of initiation and struggle to which the experimental scientist is naturally subject.

Although Bernard was skilled in experimental surgery his knowledge of chemical methods was much less developed, and his dependence upon the chemical expertise of his colleague C.-L. Barreswil was here of great importance. Barreswil had developed a method for the detection and estimation of glucose by a reduction of an alkaline solution of copper salts which, in many modified forms, had a long life.

In the first chapter of the book, entitled "Chemists, Physiologists, and the Problems of Nutrition", Professor Holmes summarises the conflicting views which prevailed about nutrition in the early years of last century, and some of the controversies in which

Dumas and Liebig figured prominently. In 1841 Magendie presented, on behalf of a commission which had been appointed to adjudicate claims about the alleged special nutritive value of gelatin, a report on 10 years of study. The French Academy were anxious to have an authoritative statement on a topic which was extremely controversial. During the previous century, and subsequently, gelatin had been regarded by some as the basic nutrient substance from which the tissues of the body are formed. The conclusion of the commission, that gelatin in itself is not adequate nourishment, evoked a storm of controversy. But the activities of the commission had an important effect on Claude Bernard since, by carrying out a number of extended feeding experiments for Magendie at the Collège de France, Bernard was initiated into the experimental investigation of animal nutrition. In that way the lectures and writings of Dumas, Liebig and others came to bear on the developing mind of the young Bernard. And the controversies that ensued induced Bernard to ally himself with those who were critical of the uninspired application of chemical methods to the solution of biological problems, although he himself made much use of chemistry in the service of physiology.

In 1848 Bernard began the series of investigations on the nature of the processes to which substances absorbed from the gastro-intestinal tract of animals were subjected during their passage through the body. The results of these investigations were summarised in Bernard's thesis for the Doctorate of Science, which was published in 1853 under the title *Nouvelle fonction du foie*. It described his perhaps most famous discovery: the glycogenic function of the liver. From 1853 onwards many publications by Bernard document his scientific development.

Bernard's *Introduction à l'étude de la médecine expérimentale*, published in 1867 as the result of a period of illness which kept him from his lecture room and research bench, gives what can be perhaps an idealised treatment of his subject and of the views that he had assumed by the time he had become an internationally distinguished investigator. Professor Holmes quotes from Bernard's writings later in life the statement that "For more than two years, at the beginning of my career, I wasted my time pursuing theories and chimeras. It is a remark-

able fact, that one imagines that the truth is in himself, and I insisted on repeating experiments which insisted on responding to me always in the same way, contrary to my views. It was not until after a long deception that I ended by reflecting and thinking that . . . my will could not change the laws of nature".

As Professor Holmes states, Bernard's strength was tied to some serious limitations, and his avenue towards greatness was at first a narrow one. There was no guarantee that his will, his insights and his technical skill would lead him to the heights. During the 1840s he was not somebody consciously advancing towards his position of the 1850s and 1860s, but one whose fate was uncertain. "The prospect of returning to Villefranche as a village doctor was as real for Claude Bernard as the prospect of ascent to the Académie des Sciences, the Académie Française and the adulation of his peers".

All who are interested in the way scientists develop will find fascinating material in this study of Claude Bernard during his early years.

F. G. Young

Scientific knowledge and sociological theory

Scientific Knowledge and Sociological Theory. By Barry Barnes. Pp.x+192. (Routledge and Kegan Paul: London, December 1974.) £3.95 cloth: £1.95 paper.

IN spite of its Germanic title and subject matter, *Scientific Knowledge and Sociological Theory* is a brief, elegantly written, and even occasionally witty discussion of the social and cultural factors affecting the development of scientific knowledge. It represents an ambitious attempt to apply the perspectives of Mannheim's sociology of knowledge to science.

Barnes rejects conventional distinctions between false beliefs, which require causal explanation, and "naturally reasonable inductions", which do not. He also rejects the definition of science as rational, rather than true, knowledge, on the grounds that there are no adequate criteria for comparing the rationality of different statements: simplicity is ambiguous, and conventional; falsifiability is impossible to establish, and rarely used in practice; and *ad hocery* is merely a derogatory term for secondary elaboration or rational modification. For Barnes, all knowledge is necessarily theoretical, for there is no direct language of observation, and theoretical acceptability is culturally conditioned. Therefore, all knowledge is equally susceptible to sociological explanation.

Normality, defined by the actors themselves, is substituted for truth or ration-

ality as the yardstick for comparing different beliefs; abnormality, rather than falsehood or irrationality, requires causal explanation. "Where actors or groups hold idiosyncratic beliefs as judged against some background of normality, some special cause or condition must be identified which distinguishes that actor or group from that background" (p. 42). Barnes, therefore, focuses upon the maintenance of normality and the development of innovation within scientific sub-disciplines. Normal beliefs are learned through socialisation into the distinctive metaphors and procedures of scientific subcultures, by following exemplary procedures, and by acquiring practical experience. Innovation occurs through differentiation, transfer from one sub-discipline to another, or simply from creativity based upon "a universal human propensity to create and extend metaphors" (p. 87). Such innovations occur partly through the intellectual evolution of conventional paradigms within science itself, and partly through the need to develop new beliefs to fulfil purposes external to science. Thus, the Scientific Revolution resulted partly from the re-emergence of Platonism, and partly from the disintegration of feudalism as a political and social system: "in the general context of post-feudal Europe, teleology had to decline" (p. 117).

The substitution of normality for truth or rationality represents only a formal, if fashionably relativist, advance upon

previous usage. What are the criteria for 'normal'? Is normality to be interpreted in a statistical or cultural sense? Are actors always conscious of the normality or otherwise of their actions? Moreover, rationality can, for 'practical purposes', provide a basis for comparing beliefs in terms of given means–ends relationships. Problems arise with the concept of rationality only when it is impossible to specify ends, that is, at the most general level. But, as Barnes points out, science consists of a number of differentiated sub-cultures, each with distinctive procedures and ends. The problems of rationality are, therefore, less than Barnes initially suggests.

In general, the sociology of knowledge provides an unsatisfactory basis for explaining scientific knowledge, for the connections between specific beliefs and the social structure remain tantalisingly vague. Barnes argues sensibly that "the sociologist should consider beliefs in terms of their functions in practical activity" (p. 39) and brings out clearly the general factors that might connect science with that activity. But the illustrations of those connections are disappointingly brief and often unconvincing: uniformitarianism as the credo of an emerging industrial order in early nineteenth century Britain and eugenics in Britain before 1914 as a response of the declining ruling class are presented as self evidently plausible examples. At the very least, some attempt at a comparative analysis of alternative reactions is required. Moreover, Barnes shows considerable sympathy for materialist explanations of scientific development (for example, pp. 149–50), but does not discuss the connection between the material bases for the production of science and the content of scientific beliefs in any detail.

The approach to sociology adopted by Barnes is explicitly 'phenomenological', although his use of the approach is valuably flexible. This involves "an appreciation of actors' normal practice as it is, and of its inadequacies as they themselves define them" (p. 43). In practice, however, the author disregards the self imposed limitations this perspective volves, and his analysis of innovation acknowledges the problematic validity of actors' own explanations. This tension, if not inconsistency, stems from the author's recognition that actors' accounts cannot in themselves provide a basis for sociological generalisation and explanation.

In spite of these unresolved problems *Scientific Knowledge and Sociological Theory* represents a valuable and interesting contribution to sociology. At the very least it provides sociologists with an unusual gritty insight into the reality of science. But I doubt whether the book will persuade scientists that sociology has much to offer them in their own 'practical concerns'. **Roderick Martin**

Finite Groups and Quantum Theory.
By D. B. Chesnut. Pp. xiii+254.
(Wiley Interscience: New York and
London, December 1974.) £7.25.

THIS book satisfies nobody. It is nicely
written and clear, but as a physicist I
could not possibly recommend it to my
students as there are no physical
applications. It is indeed remarkable
that a book on finite group theory
advertised as being suitable for
physicists contains no mention of
crystal structure This is presumably
because the author is a chemist. But
when I asked my colleagues in chemistry
for their opinion I was told that the
single chapter on the application of
group theory to chemical bonding was
far too slim and that it could not
compete with several other books in the
field.   **Norman Dombey**

Pest Control and its Ecology. (The
Institute of Biology's Studies in Biology,
No. 50.) By Helmut F. van Emden. Pp.
59. (Edward Arnold: London, January
1975.) £1.90 boards; £0.95 paper.

THIS 59-page book with its list of 17
references is intended to help "teachers
and students at school, college or
university . . . to keep abreast of recent
trends and know where the most sig-
nificant developments are taking place".
It aims to cover primarily the control
of insect pests on growing crops and,
except for occasional excursions into
other hemispheres, it devotes most
attention to British insects.

The author deals realistically with the
modern vogue for the uncritical con-
demnation of pesticides, in particular
providing yet another disparaging per-
spective of Rachel Carson's *Silent
Spring* which started so many hares
running and seemingly also set in-
numerable hounds into perpetual mo-
tion in hot pursuit.

In this book, unfortunately, the possi-
bilities for classical biological control,
in the sense of introducing natural
enemies of pests of exotic origin, are
blurred and diluted by being confused
with the mass production of agents for
use in flooding techniques. Doubtless,
this is because the author lives in a
country where there is generally con-
sidered to be relatively little potential for
the introduction of exotic agents. It is a
pity that the more generally applied
type of biological control is sold short
by mention of such remote or largely
misleading concerns as "imported para-
sitic insects may occasionally carry a
crop pathogen externally and thus bring
a new problem into the crops" and that
its use means that "control is slow, it is
not exterminant, unless 'misused', it is
often unpredictable, it is difficult and

expensive to develop and apply, and it
requires expert supervision" (pp. 19–20).

Those comments do a serious dis-
service to a flourishing branch of
entomology which is pollution-free,
involves finite energy input and may
produce valuable results lasting long
into the future.

In general, however, this book is up
to date, eminently readable and conveys
much useful information.
                              D. F. Waterhouse

Books brief

Protein–Calorie Malnutrition. (The
Nutrition Foundation: A Monograph
Series.) Edited by Robert E. Olson
Pp. xxiv+467. (Academic: New York
and London, February 1975.) $29.50;
£14.15.

ALTHOUGH this book contains many
papers of a high scientific standard it
is difficult to be totally sympathetic to
its aims or its achievements. The
majority of nutritionists now agree that
protein–calorie malnutrition (PCM)
can be prevented by eating more food
That being so, symposia on the bio-
chemical and physiological aspects of
the disease—which is essentially what
this book is—are open to a charge of
irrelevance.

Those who accept that metabolic
research is still required will find, no
doubt, that this book is a useful guide
to such projects They may be irritated
by the inclusion of a few papers with
virtually no bearing on PCM, and by the
fact that a publishing delay of two
years has made the contributions some-
what out of date. They will, however,
find it a useful text to consult, if it is at
times somewhat tedious to read.

It is a pity, though, that the editor
did not substitute some practically
useful papers for the interminable
discussion sections. Then it might have
been the important landmark that the
Nutrition Foundation claim it to be.
                              John Rivers

*Topics in Carbon-13 NMR Spectro-
scopy,* vol. 1. Edited by George C. Levy.
Pp. x+292 (Wiley-Interscience: New
York and London, December 1974.)
£9.45.

THE remarkable technical advances
made over the past five years have
allowed access to NMR spectra of
dilute spin systems to the extent that
^{13}C spectra are now commonly observed
on a routine basis. The considerable
advantages of the ^{13}C NMR technique

have engendered much interdisciplinary
interest. *Topics in Carbon 13 NMR
Spectroscopy* is a useful supplement to
existing monographs on the subject. It
contains a number of articles by well
known authors, which review aspects of
the ^{13}C NMR technique. The articles
vary from a detailed description of
theoretical work, by Professor Ditch-
field, which presents some astonishingly
accurate calculations of ^{13}C chemical
shifts (and which I found most interest-
ing), through articles on relaxation
studies (Lyerla and Levy), substituent
effects (Maciel), polymer studies
(Schaefer), ^{13}C at high-fields (Anet), to
dynamic studies (Stothers). The re-
viewers present well balanced appraisals
of their subjects up to late 1973, and
generally elucidate the shortcomings of
the methods as well as their advantages.
Regrettably, that is not the case in the
chapter on dynamic studies. In such
systems, which are non-linear, the
Fourier transform of a free induction
decay is not necessarily identical to the
continuous wave spectrum for 90° pulse
angles, although their identities become
closer as the pulse angle decreases; this
caveat should have been covered more
explicitly than by the inclusion of one
reference.

Overall, the volume has much of
interest to offer to spectroscopists and
to chemists and biochemists generally. I
look forward to the continuation of
what promises to be a stimulating and
informative series.   **J. M. Briggs**

*Flow· Its Measurement and Control in
Science and Industry.* Vol. 1, part 3:
Flow Measurement and Control. Edited
by W. E. Vannah and H. Wayland.
Pp. xliii+1049–1493. (Instrument
Society of America: Pittsburgh; Wiley:
Chichester, November 1974.) £19.60.

IN order to bring together experts in
many branches of fluid flow and to
review the most recent advances in the
subject, an international symposium
was convened in Pittsburgh in the
Spring of 1971. Some three years later
the Proceedings of that symposium have
been issued. All possible facets of
flow measurement and control have
been considered and the editors have
managed to classify the papers into
four rather vague themes entitled
"Flow characteristics", "Flow measur-
ing devices", "Flow measurement" and
"Biological fluid flows". This excellent
and ambitious book, comprising as it
does a comprehensive range of almost
200 theoretical and experimental con-
tributions, is very well produced and in
spite of the high total cost will prove to
be the ideal reference source for workers
in the entire field of fluid flow.
                              P. A. Davies

obituary

Ernst David Bergmann, professor of organic chemistry at the Hebrew University and one of Israel's leading scientists, died on April 6 at the age of 71.

Born the son of a Rabbi in Germany, he obtained his PhD at Berlin in 1933, and arrived in London during that year. There he met Chaim Weizmann, with whom he was to form a close working relationship, lasting 18 years. He left for Palestine in 1934 to head the Daniel Sieff Institute (later the Weizmann Institute), which he had helped to plan. Before accepting the professorship of organic chemistry in Jerusalem (where he resigned from the post of vice-president only last March) in 1953, he had been Scientific Director of the Sieff (Weizmann) from 1934–51. From 1953–66 he was chairman of the Atomic Energy Commission and director of defence research in the Ministry of Defence, and currently had been director of the Harry S. Truman Research Institute at the Hebrew University and Honorary President of the Ben-Gurion University of the Negev. His early studies on the stereochemistry of the Walden inversion and on polycyclic hydrocarbons, and his later work on the toxicity of organic fluorine compounds and on the role of steroids in insect growth, had gained him international fame. His research had been mainly in the fields of petroleum and photochemistry; on receiving the Solomon Bublick Prize in 1973, he had predicted the oil crisis that would beset the world, and proposed the establishment of an international agency for oil—along the lines of the International Atomic Energy Agency, in which the Arab states were participants. In recent years he had also been involved in developing contacts between the scientific and technological communities of Israel and West Germany. Among many prizes recognising his lifelong contributions to science, Professor Bergmann had received the Israel Prize for Natural Science and the Rothschild Prize.

announcements

Appointments

The Trustees of the Nuffield Foundation have appointed **John Maddox** as Director of the Foundation. **Robin Matthews** has been appointed a Trustee.

J. R. Parratt, reader in physiology and pharmacology, has been appointed to a personal professorship at the University of Strathclyde.

Birkbeck College, London has appointed **Judith Greene** as professor of psychology.

International meetings

October 2, **Current nutritional developments,** Manchester, UK (Mr J. Crowther, The Metal Box Co. Ltd, Monmouth House, Monmouth Road, Cheadle Hulme, Cheadle, Cheshire SK8 7BJ, UK).

October 6–10, **Nuclear techniques in animal production and health,** Vienna (International Atomic Energy Agency, Kartner Ring 11, PO Box 590, A-1011, Vienna, Austria).

October 6–10, **Remote sensing of environment,** Ann Arbor (The University of Michigan, Extension Service, Conference Department, Ann Arbor, Michigan, 48104).

Person to Person

London accommodation. American pharmacologist and wife, without children or pets, seek small furnished flat (1 or 2 bedrooms) in central London for October and November, 1975, while working on sabbatical leave at University College (Dr J. P. Green, 33 Avenue de Breteuil, Paris 75007, France; tel. no. 734-37-36).

Nuts. Scientist requires small samples of edible nuts without fleshy layer for photographing. Botanical description or description by use. Entry permits will be forwarded (Dr Arnold Krochmal, 13 Veterans Drive, Asheville, North Carolina 28805).

There will be no charge for this service. Send items (not more than 60 words) to Holly Connell at the London office. The section will include exchanges of accommodation, personal announcements and scientific queries. We reserve the right to decline material submitted. No commercial transactions.

October 13–15, **Electronic and aerospace systems,** Washington DC (The Institute of Electrical and Electronics Engineers Technical Activities Board, 345 East 47th Street, New York, New York 10017).

October 13–17, **Gas cooled reactors,** Jalich, German Federal Republic (IAEA/NEA International Symposium on Gas Cooled Reactors with Emphasis on Advanced Systems, c/o International Atomic Energy Agency, PO Box 590, Kartner Ring 11, A-1011, Vienna, Austria).

October 14–16, **CAMAC in computer applications,** Brussels (Dr H. Meyer, Commission of the European Communities, CRC-CBNM, Steenwegg naar Retie, B-2440 GEEL, Belgium).

October 16–18, **Platinum,** Denver (G. A. Desborough, Convenor US Geological Survey, Building 25, Denver Federal Centre, Denver, Colorado 80225).

October 20–24, **Safeguarding of nuclear materials,** Vienna, Austria (International Atomic Energy Agency, PO Box 590, Kartner Ring 11, A-1011, Vienna).

October 20–24, **Clean air and pollution control,** Brighton (National Society for Clean Air, 136 North Street, Brighton BN1 1RG, UK)

October 22, **Molecular beam–surface interactions,** London (The Meetings Officer, The Institute of Physics, 47 Belgrave Square, London SW1X 80X, UK).

nature

What can scientists contribute to arms control?

ARMS control and disarmament have had an unexpectedly active summer. Not that anybody has actually done anything about controlling arms or putting them out of harm's way, but there has been more public talk than for a long time. *Daedalus* has followed up, fifteen years on, its immensely valuable Fall 1960 issue on arms control with a Summer 1975 issue devoted to "Arms, Defense Policy, and Arms Control." The World Federation of Scientific Workers (WFSW) has recently held a major symposium ("The role of scientists and of their organisations in the struggle for disarmament") in Moscow. And the thirtieth anniversary of Hiroshima has occasioned some thought-provoking events, particularly (on BBC Television) a remarkable interview with Professor Philip Morrison who gave a lucid description of the way things were and the way people thought in those days.

There has been a nice contrast about it all; the harsh realities of Morrison, the populist aspirations in Moscow, the purring sophistications of Cambridge, Massachusetts. But have science and the scientist any future in the field of arms control? The WFSW has no doubt; "scientific workers can provide the ammunition to make the force of public opinion [for ending the arms race] overwhelming". *Daedalus* is less sure: ". . . arms control and disarmament now appear to be politically more complex than they did in 1960, while the technical aspects (of verification and the like) appear somewhat less complicated and perhaps therefore less consequential", writes Franklin Long.

The similarities between atomic weapons and arms control are striking. There was a time when certain key scientists could make direct approaches to politicians and be given a fair hearing for their views on whether and how a bomb should be built. But once government had been told how to do it, a large machine took over and nuclear weapons became no longer a crusade but an industry. So it was with arms control. There was a time when scientists had governments' ears for their views on verification; a black box here, Mr President, a satellite there, two dozen seismometers here. There were heady times, as many will testify, when it really looked as if significant nuclear limitations might have been negotiated on the basis of straightforward technical advice. No longer; the scientists gave what they knew, the machine of state absorbed it and moved on. Even arms control became less of a crusade and more of an industry.

It also became infinitely more complex, of course.

Whereas it used to be possible to talk of banning this or that, it is now necessary to deal in packages; restricting (not banning) this or that, swapping information on space medicine and wheat yields, exchanging works of art, making visas easier to get and so on. The scientist's role as an input of scientific information is a pretty minor one and at least in the nuclear field is likely to remain so.

It has also to be remembered that for every one scientist who sincerely deems it his or her responsibility to speak out in the few forums available (in the UK practically none at all, since the community of intellectuals is otherwise preoccupied) there is at least one other scientist who deems it his or her responsibility to work diligently to keep the nation's defences intact. Thus talk of Hippocratic oaths for scientists to eschew work on projects which might injure or kill humans or calls for scientists to measure up to their social responsibilities might, if taken seriously, lead to holier-than-thou witch-hunts in the scientific community.

If one cannot be optimistic that scientists *per se* have a major role to play in arms control discussions in general, there are perhaps two areas in which they can be particularly effective. The first includes weapons of the twenty-first century (perhaps, in particular, environmental weapons). Since none of these are at the research and development stage yet, there is likely to be a much greater agreement that they should be eschewed. Scientists are notoriously conservative in predicting the future of their subject, but surely if a major international effort were to be devoted to identifying danger areas, it is not inconceivable that an international consensus could be reached on control measures before rather than after the threat was in existence. Second, scientists could make a ruthless examination of the research and development aspects of every proposed arms control measure and ensure the appropriate publicity.

● Research and development is the mainspring of military improvement; beware of any agreement that leaves the laboratories intact.

● The Partial Test Ban Treaty was even used as a justification for the United States to increase its nuclear research and development activities.

● SALT has been a positive encouragement to qualitative improvement of missiles.

Nobody knows this as well as the informed scientist, and it may on occasions even be necessary for apparently benign measures to be opposed.

The East is read

To explore one important facet of the visible tip of the iceberg of Chinese science, S. Dedijer and B. Billgren ask: what was the effect of the Cultural Revolution on Scientia Sinica, *the principal journal of the Chinese Academy of Sciences? How and to what extent has politics caused it to depart from the international scientific tradition?*

AS the chief Academy journal, *Scientia Sinica* occupies a unique position both inside and outside China. Although, as a result of the Cultural Revolution, the journal ceased publication for seven years in 1966, it is in China the standard bearer of science and the most prestigious channel for scientific communication. Abroad it is the official broker of knowledge on the state of China's science and stakes out China's claims amongst world scientific competition. Thus, focusing on this very visible yet little studied face of Chinese science, one learns not only about changes from 1965 to 1974, but gains an insight on the present state of the scientific community in China.

Our basic materials are the four v o l u m e s ((XIV–XVII: 1965–1974) with 28 issues and 405 articles) of the foreign language edition of *Scient. sin ,* identical to the Chinese edition (we compared two 1973 issues of each). To this material we applied a number of simple qualitative and quantitative indicators for the international scientific tradition and its change by politics.

Tradition: (1) the journal as communication channel; (2) absence of politics in articles; (3) structure of the journal; (4) structure of articles; (5) science norms; (6) style; (7) priority protection.

Changes by politics in: (1) editorial policy—including "mass science" and "serve the people"; (2) political content of articles; (3) authorship of articles; (4) research programme in journal; (5) language of articles; (6) institutional changes; (7) origin of references; (8) rapidity of publication.

An analysis of the 405 articles shows that all overt political statements in them can be classified under one of the following categories—

● Mao citation: "Chinese medicine is a great treasure which should be investigated and further developed for the welfare of mankind (quoted twice during 1973).

● Political statements: "Since the founding of New China and under the correct leadership of the Chinese Communist Party" in "Cure of Choriocarcinoma and Chorioadenoma Destruens Chemotherapy" (No. 2, 297; 1973).

● Sentences with overt marxist-maoist orientation: "The correct road of dialectical materialism can be only assured by going deeply into the essence of this problem" in "Crustal Structure and Crustal Movement" (No. 4, 520; 1973).

● Policy Statements: "We are still a developing country. Steps have just been taken to study the aforementioned significant theoretical problems of science" (such as pulsars, quasars and background cosmic radiation), in "Heliocentric Theory in China" (No. 3, 376; 1973).

The political content of articles is measured on a 0, 1, 2 scale: 0 for articles without a single overt political sentence, 1 for one or two such sentences, 2 for more than two.

At the beginning of the Cultural Revolution in 1965–66 there were strong hints in the literature that the publication of scientific journals was anti-revolutionary. The most revolutionary impact of the Revolution on *Scient sci.* was that it ceased publication for almost seven years together with all the other journals, except *Chinese Medicine,* which continued until 1968 in a highly political form. With the renewed publication of the foreign and Chinese editions of *Scient. sin.* (and 19 other journals) in 1973, China returns not only to the 300-year-old international tradition but also to its pre-1949 tradition. For *Scient. sin* is the new name given in 1955 to the Chinese Academy journal *Acta Scientia Sinica* begun in 1952, which in its turn succeeded *Science Record,* the main Academy journal in 1942–45 under Ch'ang Kai Shek's government The editor of that, as well as of *Acta scient. sin.* from 1952 was the Chicago

trained physicist Y. H. Woo, vice-president of the Academy from 1949 until after the Cultural Revolution.

Exactly resembling the corresponding Western academy journals and its Chinese predecessors, *Scient. sci.* throughout its history up to issue No. 2 of 1974 consisted exclusively of articles communicating to the Chinese and the world scientific community the latest research results in "pure science, technology, agriculture and medicine wtih emphasis on basic theoretical research". Several scannings of the 405 articles show that all those in 1965–66 and 74% of those in 1973–74 follow the Chinese and Western tradition: they contain not a single overt political sentence.

All the 405 scientific articles from 1965 to 1974, even those with political content 2, have like their Chinese predecessors the traditional article structure. The title is followed by the name of the author(s), the institution(s), "date received", "abstract", "introduction", followed by the traditional sections "method", "experimental results", depending on the subject. All the articles in *Scient. sin.* close as elsewhere with sections "conclusion" or "discussion", "acknowledgements" and "references".

The fact that following the international tradition all the 1973–74 articles record the date the article arrived on the editor's desk is related —as are all other components of the standard article structure—to specific socia-cognitive components of the research process. This "date received" usage has at least two functions: it stakes a scientist's claim to priority in his field, and it measures the speed of publication. Encouragement of international competition in science and technology has been a constant policy of the Chinese leadership. Mao, Lin Piao, Liu Shao Chi, and Chou En Lai have continuously stressed that "the country will certainly catch up with and surpass advanced world science levels in the not distant future" (*Scient. sin.,* No. 2, 279; 1973). Of the 235 articles in 1965 only one was published within a month of receipt. This was the most significant Chinese contribution to world science since 1949; synthesis of the insulin molecule by a group of 21 authors (*Scient. sin.,* No. 11, 1965). The three articles on the subject in 1973 and the two in 1974 were also published more rapidly than the average waiting time of six months. The only two articles in 1973–74 published within two months of receipt had definite political overtones, both were results of collaboration between an American (of Chinese descent) and a Chinese scientist.

A detailed study of the style and content of articles in 1973–74, even the

most political, shows a consistent effort to be impersonal and objective in style. The authors express themselves with the usual professional researcher's methodological reservations and scepticism. They relate their ideas and results to those of others and to knowledge already confirmed. There is a continuous professional striving toward a scientific approach, results and conclusions. Hence all the articles—even the most political—follow at least three of Merton's "institutional imperatives" of professional ethics of science. The 300 of 405 1973–74 scientific articles with no political statements can be said to follow the fourth, the "disinterestedness" imperative, they contain no explicit statements on the social significance of the reported research either in "serve the people" language or any other.

Only two out of 93 articles in 1973–74 are directly related to the important goal of the Cultural Revolution—"science by the masses". One in 1973 is by a peasant on "Using Chairman Mao's Philosophical Thinking to Guide Scientific Experiment in Peanut Cultivation". The second in 1974 is by a "worker-instructor" on "A Mathematical Analysis of Involute Gear Periodic Errors". Both articles—one with a political content of 2, and the second with 0, follow closely the traditional article structure.

The political content of articles and of the journal are shown in Fig. 1. As seen, there was no politics in 1965–66 articles. During 1973, 56% (24 out of 43) had no political content compared with 86% (43 out of 50) in 1974. From 1973, issue No. 1, the political content of articles decreased continuously and in 1974 it amounted to one or two points per issue. These "political bits" are to be found with few exceptions in the introduction or conclusion sections, and many are repeated in several articles giving the impression of being insertions by the editors.

With No. 2, 1974, two sorts of purely political articles began to appear. The first, at the beginning of each issue are poltical editorials criticising Lin Piao, Confucious and "the theory of innate genius". The second, placed at the end of the issue are philosophical "Studies on History of Natural Sciences". Such articles (Fig. 1) take about 10% of the pages of the journal. Thus it seems that the political bias of purely scientific articles is being replaced by a more pronounced political editorial policy with the double goal of guaranteeing the political correctness of the journal and the ideological education of the readers.

Figure 2 shows some significant changes, probably politically induced, in the articles in 1973–74 as against

Fig 1
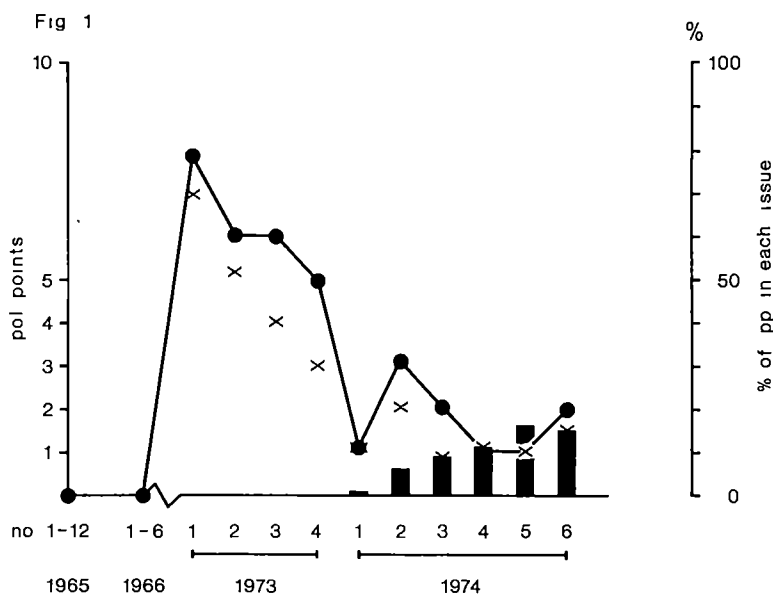
X No of articles in given issue cf Sc S with pol points ●

Fig 2   Changes in Sc S

1965–66   :   1973–74
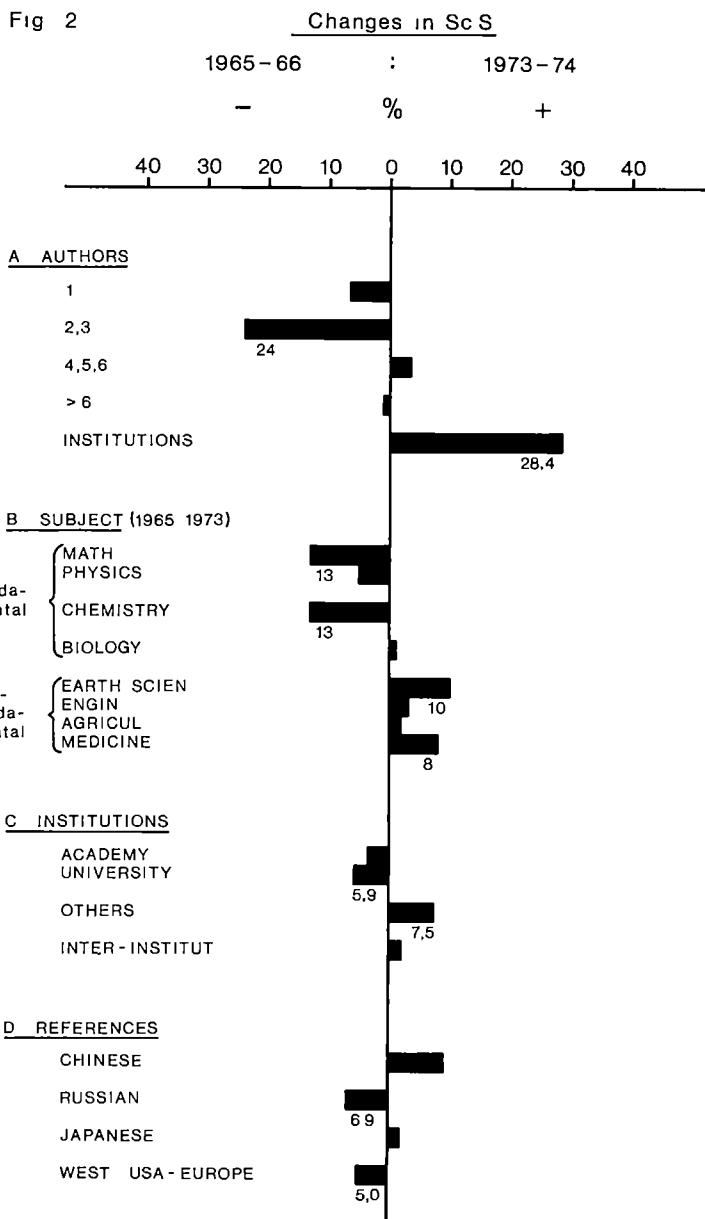
1965–66. From *a* we note a significant decrease of articles by 1, 2 or 3 authors and an increase in collective authorship giving only the name of the institution(s). This may turn to be temporary for it makes difficult the communication a m o n g researchers working on the same subject. Figure 2*b* shows a shift between 1965 and 1973 from the theoretical work in fundamental to non-fundamental sciences. It seems that *c* shows no significant shift in the pattern of institutions performing the work except for the decrease of that in universities. As regards the national origin of references (a total of 3,533 in 1965–66 against 1,242 in 1973–74) we note in a 6.9% decrease of Russian and 5.9% of Western, the latter amounting to 60% of all 1973–74 references. In 1965–66 there were 22 articles in Russian and 9 in French compared with 1 and 2 in 1973–74.

Although our method is relatively simple, it seems to us that a more refined one would not lead to radically different results. We conclude, first, that our results from *Scientia Sinica* do not support such widespread general conclusions by visitors, as for example by A. Peyrefitte, namely, that in the Cultural Revolution "Scientists have been dealt a body blow. The functioning of research has been revolutionised to a degree which is hard to imagine, except, however, for national defence projects".

We find quite the contrary situation. Except for a very significant quantitative decline (312 articles and notes in 18 issues in 18 months of 1965–66 against 93 articles in 8 issues of 24 months of 1973–74) our results indicate that the goals of the Cultural Revolution are not reflected to any significant degree in the 1973–74 *Scientia Sinica*. All our evidence shows that the science China is most proud to show the world is still done entirely by professional scientists, who for their ideas and research problems still turn, as in the rest of the world to national and international science itself and not to the masses. This points to the existence of a scientific community in China which functions by the same traditions, norms and usages as in USSR, USA and Europe. We also note that in the period studied, the Chinese have not made any new innovations in this tradition which are visible in *Scientia Sinica* except for the radical increase of "collective authorship" of dubious value. Consequently we conclude that—with the exceptions noted —the *Scientia Sinica*, has followed in 1973–1974, as throughout its history, the international tradition of science, according to chairman Mao's directive "Make the past serve the present and foreign things serve China".

Barefoot in the field

Though a thorough study of the barefoot doctor system has yet to be carried out, there is no doubt that it represents the first successful, large-scale attempt at the delivery of total health care in a developing country cut off from Western civilisation. Alexander Dorozynski discusses the system by which, in the space of a few years, China has managed single-handedly both to overcome chronic malnutrition and to bring under control most of the epidemic diseases which have plagued it in the past.

THE really significant developments in health care delivery in China have taken place during the past 10 years, since 1965, when Chairman Mao issued the "great call": "in medical and health work, put the stress on the rural areas." The results, although now taken for granted, have been amazing and unequaled anywhere else.

Child mortality has been reduced in a spectacular fashion, and something that seems to be a nationwide family planning programme has been set up. In a truly revolutionary process, the Western 'medical monopoly' has been overturned, yet its achievements have been incorporated into a truly national medical system that has drawn both from the experience of countless generations of traditional practitioners, and from modern science.

The term "barefoot doctor" is now known the world over. It seems that it was first used in the Chiangchen People's Commune on the outskirts of Shanghai in 1965, where a medical team started teaching young farmers to perform some medical tasks. These farmers continued to work in the fields, but also cared for their fellow farmers, who affectionately called them "barefoot doctors". A Chinese delegate at the 27th World Health Assembly has said that since then, about 1 million barefoot doctors, of different levels adapted

to the needs of their community, have been trained in China.

In developing countries, medical doctors tend to congregate in the cities, largely leaving rural areas unattended, with the exception of occasional visits or vaccination campaigns. In China, many mobile medical teams tour the countryside, transporting with them especially designed equipment that allows even major surgery to be performed 'in the field'. They attend to the most serious cases, teach, and give refresher courses to barefoot doctors.

An important aspect of this programme was pointed out at the World Health Assembly by Dr Wang Kuei-chen, a 37-year-old barefoot doctor— "There is no barrier between the doctor and the patient"—white coats, impersonal offices, gleaming instruments and the medical language, all of which often contribute to isolate the patient in the West, play no part in this patient–doctor relationship. Barefoot doctors, she pointed out, are called 'doctors', not paramedicals or auxiliaries; but the relationship is close enough, she said, for her usually to be called "little sister" or "little daughter".

The major concern is with preventive medicine, and this may well be the principal reason of the system's success. Basic preventive medicine, immunisation, health education, well-baby clinics, and surveillance of communicable diseases are enough to cut down radically the incidence of the most common illnesses in rural areas. Labour-intensive techniques have been used effectively against epidemics. For example, millions of schistosomiasis-bearing snails have been picked by hand and destroyed, and there have been similar campaigns against bedbugs, mosquitoes, rats, and flies. As one medical professor has put it: "Each of us, regardless of whether he was a janitor or university president, had to carry around a big fly swatter and a vengeful lust for dead flies. Everyone had his or her quota to fulfil by the end of the week."

This did not exclude curative medicine. The recognition and treatment of diseases such as respiratory infections and diarrhoea, which are among the most widespread causes of infant mortality, is in most cases a simple task, which requires neither a medical degree nor eight years of medical training. In such instances barefoot doctors practise what is referred to as "the New Chinese medicine", an integration of western and traditional procedures including acupuncture, herbal treatment, modern drugs and sometimes relatively complicated surgical procedures such as appendectomies. According to most reports, female barefoot doctors also routinely perform abortions, using the aspiration method. □

international news

THE Atomic Energy Commission (CEA) in France has reached the most important turning point in its history since its creation in 1945. On August 6, in a decision running counter to demands expressed by industrial syndicates that the total nuclear sector be placed under public responsibility, M Valery Giscard d'Estaing approved the separation of the production side of the atomic energy industry in order to form a limited private company, a wholly owned subsidiary of the CEA.

In practice, the Directorate of Production constitutes a homogeneous entity with a wide range of activities, providing the military sector with strategic nuclear equipment and the public sector with all the services required for the fuel cycle—ores, enrichment, fabrication of fuel, treatment of irradiated fuel, storage of waste and so on. With the acceleration of the French nuclear programme, the resources of this department of the CEA have grown rapidly during the past few years, and in 1974 it even managed to defray 59.2% of its own costs. Because of budgetary and human imbalance in this sector as compared with the whole of the CEA, it follows quite naturally that it should become an industrial company.

At the same meeting, President Giscard d'Estaing agreed that the CEA, through the new company, should buy a stake in Framatome, now the sole construction company for nuclear reactor steam generators in France. Since 1973, two companies making nuclear generators have been competing for the French market, Framatome (a multinational corporation, 51% of which is owned by Creusot-Loire, 45% by the American Westinghouse group, and 4% by the Empain-Schneider group) and

Restructuring the French nuclear industry

from the staff of La Recherche

the General Electricity Company (CGE). The former is licensed by Westinghouse to use the pressurised water method. The government, going back on its two-year-old decision to go nuclear, decided at the beginning of the year to slow down the projected generation of nuclear energy. Since then, profiting partly from its former position in the French market and partly from a larger slice of the French industrial cake, Framatome has come through as the sole constructor of nuclear reactors. The government now has to negotiate the purchase of some of the interests of the American company. Although nothing is known about the talks in progress with Westinghouse, it seems that it might be persuaded to keep only an 11–12% stake in Framatome, which would give the CEA a minority block (with veto power) within the company.

Another industrial rearrangement adopted at the August 6 meeting is the setting up of a common structure between Framatome and the two companies making turbo-alternators in France, CGE and CEM (the latter a subsidiary of the German-Swiss group Brown-Boveri). As a result they can offer the world entire nuclear reactors, complete and ready to go.

The official arguments behind the new decisions are clear. The arrangements provide independence from the American license, gallicise the electro-

nuclear industry, create a powerful, competitive company to compete in the world construction market, and provide control over exports.

Industrialy, the arguments are also very clear, but present distinctly divergent points of view, reflecting three main lines of thought. First, in gallicising American technology, and thereby raising France to the rank of a partner, the result will be to make available the multinational Empair-Schneider-Westinghouse group the scientific, technical and financial potential of a public body.

Second, the creation of a production subsidiary actually prolongs the gradual breaking up of the CEA, already initiated several years ago in the field of computing. The government has formed a limited company with state capital from a body which, having obtained resources for 20 years from the public sector, had arrived at a peak as regards work done on the fuel cycle Though the principal client in France will still be the EDF, the French electricity authority, there is no benefit, either technical, industrial or even commercial, in forming this subsidiary company, unless it is derived from a progressive entry into the multinational market and from the involvement of other private groups, in particular of the Pechiney–Ugine Kuhlmann group, which is already a rival in the fuel cycle area.

Finally, the industrial groups view this gradual movement towards the formation of private companies with concern, lest the laws governing profit should impinge on production matters. In a phase of fast industrial development, where considerations of profitability and production predominate, the position of workers will remain at risk.

A DISCOVERY which, if confirmed, would rank as a major milestone in physics and which would upset some of the most sacrosanct physical equations, was reported in the United States last week. Physicists at the University of California at Berkeley and at Houston University believe they have detected a magnetic monopole in the upper atmosphere.

The evidence comes from a track made in a package of plastic sheets and a photographic plate flown from a balloon at 130,000 feet in 1973. The belief is the track was made by a magnetic monopole with a magnetic

charge of 137 and a mass of more than 200 times that of a proton, which was travelling toward the Earth at

Monopole evidence

about half the speed of light.

One of the team, P. Buford Price said last week that "from any piece of our evidence you might conclude a different particle passed through our detector. But all the findings put together force the conclusion that it was

a monopole. There is no other known explanation".

First predicted by Dirac in 1931, magnetic monopoles have been hunted intensively and their possible existence has been hotly debated ever since. Physicists have anticipated confirmation of their existence with mixed feelings, since although they would add symmetry to the world of particle physics—they would be the magnetic equivalent of electrons and protons—their existence is incompatible with the Maxwell's equations, which have stood since 1865 as the basic equations of electromagnetism.

THE success of the Ariel-5 satellite has produced an unfortunate outburst of parochialism in some sections of the British press, with comments such as "Britain leads the world in X-ray astronomy" and "most discoveries are British" recurring among reports of the latest discovery. This may be true at the present moment, since the US Uhuru satellite is no longer effective, plans for a European X-ray astronomy satellite have yet to be finalised and clearly the UK national press finds the Netherlands too small to notice. But the reaction is a particularly unfortunate one in view of the great importance of international cooperation in X-ray astronomy, and the great success of that cooperation so far.

Data from the pioneering Uhuru satellite, for example, were made widely available outside the circle of US experimenters directly concerned with its design and operation; the widely publicised Copernicus satellite combines both US ultraviolet telescopes and UK X-ray detectors: and indeed Ariel-5 itself carries a US experiment, and was, come to that, launched by a US rocket. The series of letters beginning on page 628 of this issue gives some idea of the extent of international cooperation in this sophisticated area of research; an even better indication is provided by the four letters on pages 107–112 of this volume, in which groups from three countries using observations from four satellites and from the ground combine to describe a change in the X-ray and radio emission from the source Cygnus X-1.

Of course there is an air of friendly rivalry between the different groups, especially in the urge to publish observations of an exciting new discovery first; but everyone involved in X-ray

Astronomy in Britain

astronomy is well aware of the need to make the best use of the limited funds available. This will not be achieved if we move towards a situation where the rivalry becomes real and deep, with data being hugged close to the chests of the discoverers while they indulge in mutual claims that "my satellite is better than yours". It is to be hoped that the discoverers of exciting new phenomena in space will in future, when they announce how remarkably clever they have been to find anything at all, encourage the listening reporters to present a balanced view of the remarkably succesful international collaboration behind the whole story. To many people, it is more significant to learn that scientists from different parochial groups can work together in effective harmony than to learn that something has been discovered and that no one quite knows what it is, but at

least it was a British discovery.

● Minor Planet Circular 3827 of the International Astronomical Union announces the names accorded to a number of Minor Planets discovered by Dr L. Kohoutek with the 80-cm Schmidt Camera of the Hamburg Observatory, Bergedorf. A singularly appropriate one is MP 1896, named Beer, in honour of Dr Arthur Beer, formerly of the Cambridge Observatories, who recently celebrated his 75th birthday. The citation refers particularly to his 20 year editorship of the series *Vistas in Astronomy,* first produced in 1955 in honour of Professor F. J. M. Stratton, but continued as a serial publication that is highly regarded by astronomers for the wide ranging nature of its articles, essays and reviews. Dr Beer joins the small number of astronomers in England with a planet named after them in their lifetime—others were Stratton, and Dr G. Merton of Oxford.

The same Circular names planets 1897–1899 after J. R. Hind, the English astronomer who discovered 10 minor planets in the middle of last century, and after P. H. Cowell and A C Crommelin, distinguished in this century for their contributions to computational methods in minor planet and comet theory. Together they investigated the orbit of Halley's comet and identified its apparitions back to 239 BC.

UNDER a new agreement between the United States and Israel a water desalination project likely to cost $55 million has just been started. A test module is to be built at the Mediterranean port of Ashdod, about 40 km south of Tel-Aviv, to be followed by a prototype plant with a desalting capacity of 10 million gallons a day, linked with the local Eshkol power plant. America's contribution will amount to $20 million.

American interest in Israeli desalination has a long history. Section 219 of the Foreign Aid Bill approved by Congress in 1969 provided for the joint participation of the U.S. government and the State of Israel in the development of a large desalination plant, including the construction of a prototype for such a plant and its test operation. For this the sum of $20 million dollars was allotted by Congress.

In February 1971 the Israeli National Council for Research and Development (NCRD) presented to the US, on behalf of the Israeli government a detailed draft of a proposed Multi-Effect Distillation process (MED), developed by Israel Desalination Engineering Ltd (IDE), and backed by NCRD since 1970 as

Israel and US in water deal

from Kapai Pines, Jerusalem

probably the best desalination system in the world.

In November 1972 a memorandum of understanding was signed between both governments to construct an MED prototype plant at Ashdod, and on May 13 this year the US Secretary of the Treasury and the Israeli Minister of Finance signed a joint statement in Washington while meeting at a US–Israel Joint Committee for Investment and Trade. They said that the proposed joint water desalination project had undergone a lengthy period of evaluation, and they agreed that it was now feasible to proceed with the arrangements for the design, construction and initial operation of a large-scale prototype plant and to negotiate a technical agreement subject to the necessary consultations with Congress. Subsequently, a US technical mission came to Israel, and on May 21 a joint agreement was negotiated between the two countries to carry out the project.

A small MED pilot-plant inaug-

urated near Eilat in June last year, produces about 1 million gallons of potable water per day. This water is integrated into the regular water supply system of Eilat, and the technology of this plant forms the basis for the proposed large-scale plant in which the Americans are interested. The new distillation process is said to cut the cost of desalted water from 60 American cents per 1,000 gallons in other distilling processes to less than 24 cents. One of the distinguishing advantages of the IDE process is that it can efficiently employ low-pressure, low-temperature steam, while other distillation processes require higher temperatures for efficient operation Furthermore, the overall process is characterised by maximum energy recovery and minimal waste.

Another distinguishing feature resulting in economic advantage involves the use of aluminium tubing rather than copper in the fabrication of heat exchangers. The use of the aluminium is made possible by the relatively low temperature of the process, and also results in a substantial savings, since heat exchangers comprise a major cost-element in plant construction.   □

news and views

Tumour virus proteins at the cell surface

from Reinhard Kurth

THE enveloped RNA-containing viruses are oncogenic for a wide variety of species, of which the avian, murine and feline systems have received considerable attention. Following the discovery that these viruses replicate by reverse transcription by way of a DNA provirus (Baltimore, *Nature*, **226**, 1209, 1970; Temin and Mizutani, *Nature*, **226**, 1211; 1970), it was shown by nucleic acid hybridisation that all cells of the species tested contained chromosomally integrated provirus information. Depending on the genetic background of the host, the interaction between endogenous virus and host cell can lead to different states of virus expression, ranging from complete repression, partial expression of some virus polypeptides (VPP) to complete particle production. Silent endogenous viruses can often be activated by (mis-) treating cells with chemical or physical agents (Lowry *et al.,* *Science,* **174**, 155; 1971; Aaronson *et al.,* *Science,* **174**, 157; 1971). The question why these and possibly all animals (and humans?) harbour endogenous virus genomes is a mystery, which may now be gradually resolved.

If endogenous virus genomes were consistently silent with no potentially harmful effects, it would be understandable that no selective evolutionary pressure has operated against them. As mentioned above, this does not seem to be the case: provirus information can be translated into VPP. Gross obtained evidence a long time ago that a vertically transmitted and *in vivo* activated murine leukaemia virus was indeed oncogenic (*Proc. Soc. exp. Biol. Med.,* **76**, 27; 1951). Recent work by Aaronson's group seems to indicate also that *in vitro* activated endogenous viruses can be oncogenic (Stephenson *et al*, *J. Virol.,* **13**, 237; 1974; Greenberger *et al., Cancer Res.,* **35**, 245; 1975). Why then has natural selection failed to eliminate this information from the cell?

The answer could lie in some new data which indicate that endogenous viral information may also become activated *in vivo* under specific and so far little characterised circumstances to play a part in differentiation and/or in the prevention of oncogenesis by exogenous oncorna viruses. In such

specific instances, the expression of endogenous viral information may confer a selective advantage for the host.

Progress in the understanding of virus–host cell interactions has advanced with the isolation of the major structural VPP (see table). The individual VPP are biochemically and immunologically distinct, the only cross-antigenicity existing between the glycosylated envelope proteins of a given virus strain. On the other hand (glyco-) proteins of the same size are biochemically very similar and cross-antigenic for virus strains of the same or related species. They exhibit both group-specific and interspecies-specific antigenic determinants in addition to their distinct virus type-specific antigens (reviewed recently by Bauer, *Adv. Cancer Res.,* **20**, 275; 1974).

Plasma membrane of tumour cells

For the sake of this discussion, it is necessary to distinguish between VPP inserted into the surface membranes of uninfected and of oncorna virus-infected cells. For more than a decade it has been known that virus envelope glycoproteins (gp85 or gp69/71) are often inserted into the membranes of oncornavirus-infected cells, which is not too surprising as these viruses bud from the host cell membrane. What is unexpected, however, is the more recent finding that p30, the major structural internal protein of oncornavirus particles, can also become inserted into the plasma membranes of exogenously infected cells (Ferrer, *Int. J. Cancer,* **12**, 378; 1973; Yoshiki *et al., Proc. natn. Acad. Sci. U.S.A.,* **70**, 1878; 1973; and *J. exp. Med.,* **139**, 925; 1974). These first observations have now been confirmed and extended by several other laboratories to show that in addition to the large envelope glycoproteins, p30, as well as the small VPP p15, p12

and p10 can be expressed either alone or in different combinations on the surfaces of a wide variety of tumour cells (Friedman *et al., J. Virol.,* **14**, 1126; 1974; Ikeda *et al., J. Virol.,* **14**, 1274; 1974; Billelo *et al., Proc. natn. Acad. Sci. U.S.A.,* **71**, 3234; 1974; Moroni *et al., Intervirology,* **3**, 292; 1974; Knight *et al., Int. J. Cancer,* **15**, 417; 1975; Del Villano *et al., J. exp. Med.,* **141**, 188; 1975; Hunsmann *et al., Virology,* in the press; Schwarz *et al., Virology,* in the press).

Furthermore, some chemically induced mouse tumours can express gp69/71 and low levels of p30, p15, p12, and p10 at their surface (Grant *et al., Proc. natn. Acad. Sci. U.S.A.,* **71**, 5037; 1974; Ikeda *et al., J Virol.* **14**, 1274; 1974; Yoshiki *et al., J. exp. Med.* **139**, 925; 1974). In these instances the VPP must be coded by activated provirus. These observations extend earlier data on endogenous virus activation by physical and chemical agents (reviewed by Todaro, *Persp. Virol.* **8**, 81; 1972).

On the other hand, transformation events do not always lead to the insertion of viral products into the plasma membrane (Rapp *et al., Virology,* **65**, 392; 1975) and we know little about what (genetic) factors determine the transformed phenotype. Qualitatively similar results have also been obtained in the feline (Yoshiki *et al., J. exp. Med.* **139**, 925; 1974; Ikeda *et al., J. Virol .,* **14**, 1274; 1974; Essex, personal communication) and the avian (Kurth and Bolognesi, unpublished) oncornavirus models.

Plasma membrane of normal cells

The detection of non-glycosylated VPP on the transformed cell surface cannot easily be explained. Why are they there? What is their function, if any?

Major structural polypeptides of oncornaviruses							
Species		Virus polypeptides (VPP)					
Avian*	gp85‡	gp37	p27§	p19	p15	p12	p10
Murine*	gp69/71	gp45	p30	p19	p15	p12	p10
Feline†	gp100　gp70		p30	p21	p15	p11	p10

*,† Data from: *August, Bolognesi, Fleissner, Gilden, and Nowinski, *Virology,* **60**, 595; 1974. †Essex, *Adv. Cancer Res.,* **21**, in the press.
‡gp85 = glycoprotein of molecular weight 85,000.
§p27 = non-glycosylated protein of molecular weight 27,000.

The attempt to find a general explanation is further complicated by the recent discovery that certain normal, uninfected cells also synthesise VPP which are expressed in their plasma membrane.

Experiments with tumour cells and monospecific sera directed against individual VPP have often given background reactions with normal mouse, rat, cat or chicken fibroblasts (Grant, Ikeda, Yoshiki, cited above; Hunsmann *et al., Virology,* in the press; Schwarz *et al., Virology,* in the press; Kurth and Bolognesi, unpublished; Hogg, personal communication). Some groups decided to neglect the normally weak antiserum reactivities by preabsorbing their sera on normal fibroblasts. This approach may be justified, as the purified VPP used for immunisation may have been slightly contaminated with normal cellular material. It is, however, just as conceivable that normal fibroblasts express some VPP, perhaps in a form structurally modified by the host cell. Data obtained in the avian oncornavirus system seem to support the latter interpretation; gp85 and p27 seem to be present in comparatively high quantities in the membranes of most if not all uninfected chicken fibroblasts derived from embryos of various flocks (Smart, Bosch, Kurth and Bolognesi, unpublished).

A role in differentiation?

It is not surprising that normal lymphoid tissue from high leukaemia mouse strains express gp69/71 and p30 in their membranes (Yoshiki *et al, Proc. natn. Acad. Sci. U S.A.* **70,** 1878; 1973; Kennel *et al., Virology,* **55,** 464; 1975; Del Villano *et al., J. exp. Med.* **141,** 172; 1975), as these cells might be considered premalignant. Less expected, however, are the recent results obtained by the virologists of the Scripps Clinic: they now find gp69/71 not only on mouse lymphoid cells, but also on certain foetal and adult tissues, notably epithelial cells (Lerner, Wilson, Del Villano, McConahey and Dixon, unpublished). The data indicate that the membrane expression of endogenous VPP might follow a precise pattern with respect to tissue distribution and kinetics of appearance/disappearance. It has been suggested repeatedly for various animal model systems that morphogenesis and subsequently the maintenance of a differentiated state could be mediated (among other factors) by cell surface receptors (for reviews see *Humoral Control of Growth and Differentiation,* Academic Press, New York, 1973). If endogenous VPP are indeed among those receptors possessing a transmitter function in the exchange of information between cell and environment,

they would have a role in the induction and maintenance of established morphogenetic patterns. Such a role could explain the positive selective advantage in having potentially oncogenic endogenous virus genomes integrated into the cellular genome.

At this point, one is left with a complex situation. On one hand, VPP coded by endogenous or exogenous oncornaviruses can become expressed on the transformed cell surface and could therefore be considered as tumour-specific antigens. On the other hand, certain normal tissues also seem to express endogenous membrane VPP. The picture is further complicated by the concomitant presence of natural antibodies directed against endogenous VPP (Hanna *et al., Cancer Res.* **32,** 2226; 1972; Ihle *et al , J. exp. Med.* **138,** 194; 1973 and *J. exp. Med.* **139,** 1568; 1974; Lee *et al., J Virol* **14,** 773; 1974; Aaronson and Stephenson. *Proc. natn. Acad. Sci. U.S.A.* **71,** 1957; 1974). This situation is reminiscent of tumour-bearing animals in which tumours and their specific antigens coexist with anti-tumour antibodies and lymphocytes.

Anti-tumour response

Could VPP on tumour cells be used as tools in vaccination or immunotherapy? This seems unlikely as immunisation with isolated VPP could greatly enhance the risk of autoimmune diseases or glomerulonephritis. In fact, Hanna and coworkers (*Cancer Res.* **32,** 2226; 1972; *J. natn. Cancer Inst.* **52,** 117; 1974) have described a correlation between the development of glomerulonephritis and the persistence of high titres of natural antibodies.

In contrast, there are experimental situations in which the anti-tumour response is clearly mediated by VPP located in the transformed cell surface (Knight, Mitchison and Shellam, *Int. J. Cancer,* **15,** 417; 1975; Gorczynski and Knight, *Br. J Cancer,* **31,** 387; 1975) or in which immunisation with exogenous gp69/71 leads to significant anti-tumour protection (Hunsmann, Moenning, and Schäfer, *Virology,* in the press). A comparison of the antigenic determinants accessible to antibodies on the cell surface (not necessarily of the VPP molecules in the cell membrane) with antigenic sites in the viral particle may resolve these discrepancies. There is preliminary evidence that endogenous VPP in the cell membrane may have undergone somatic cell modification (Del Villano *et al., J. exp. Med.* **141,** 172; 1975; Tung *et al., J. exp. Med.* **141,** 198; 1975). Thus it may turn out that the anti-tumour response is (predominantly) directed against antigenic sites restricted to exogenous VPP of the

transforming virus which are not concomitantly expressed by endogenous membrane VPP in normal cells For both theoretical and practical reasons, this problem deserves further clarification.

Control of VPP expression

A final question concerns the control of endogenous virus expression, especially in differentiating tissue. There are at least two model systems where this question can be studied further. First, recent work from Boyse's laboratory at the Memorial Sloan–Kettering Cancer Center proved convincingly that the G_{IX} antigen on normal thymocytes of certain mouse strains (Stockert, Old and Boyse, *J. exp. Med.* **133,** 1334; 1971) is biochemically and immunologically related and possibly even identical to Gross leukaemia virus gp69/71 (Obata *et al., J. exp. Med.* **141,** 188, 1975; Tung *et al., J exp. Med.* **141,** 198, 1975). The second model exists in the avian system, where the helper activity of normal chicken fibroblasts to complement the defective Bryan high titre strain appears to be correlated with the synthesis of a chicken helper factor thought to be gp85 (Payne and Chubb, *J. gen. Virol* **3,** 379; 1968; Weiss and Payne, *Virology,* **45,** 508; 1971).

In general, the membrane insertion of individual VPP seems to be a non-coordinate event; different combinations of VPP have been detected on normal and transformed cells. Their expression appears to be governed by autosomal dominant genes (Strand, Lily and August, *Proc natn Acad. Sci. U.S.A.* **71,** 3682; 1974), just as in the G_{IX} and chicken helper factor models. (For recent reviews see Lilly and Pincus, *Adv. Cancer Res.* **17,** 231; 1973; Weiss, *Persp. Virology,* **9,** 165; 1975.)

It has been a drawback for progress in cell surface immunology, including tumour immunology, that many of the groups involved were predominantly occupied with the mere description of the distribution of cell surface moieties. Very few attempts have been made to investigate their function, for example with respect to enzymatic activities or transport processes. Since it is now possible to purify endogenous or exogenous VPP in milligram quantities, one may soon be able to test them for possible functions which may explain their cell surface location Additional data on the distribution of endogenous VPP in different organs and the kinetics of their appearance and disappearance in differentiating tissues may establish further associations between the expression of a virus-related phenotype and the process of cellular differentiation. □

More RNAs for oocytes

from Pamela Hamlyn

ALTHOUGH *Xenopus laevis* oocytes are interesting in themselves as a means of studying gene action, their more familiar role is that of an assay system for the translation of injected messenger RNAs. They have many advantages for this purpose, not least their ready availability—several thousand mature oocytes from one adult female. (Compare this with scanning the shelves of health food shops for just the right wheat germ.) Injected messenger RNA is translated with a high efficiency, globin mRNA being translated at nearly the same rate as in intact reticulocytes. Because of this very high efficiency only minute amounts of mRNA are required to produce detectable amounts of protein product and consequently mRNA activity can be detected even when crude RNA fractions containing very little mRNA are injected into oocytes.

van der Donk made use of this property in some experiments that are reported in this issue of *Nature*, **256**, 674; 1975). He is interested in the mechanism by which the style of *Petunia hybrida* is able to recognise and to differentiate between its own pollen and that of another plant. (Pollen tubes only grow after cross-pollination.) Total RNA extracted from self-pollinated styles stimulated

A hundred years ago

AMONGST the objects which have been recently added to the galleries of the Paris Industrial Exhibition of Geography, and are attracting public notice, we may mention a collection of French birds exhibited by M. Bouvier, the collection of apes from the Gaboon, by the Marquis de Compiègne, and a number of antediluvian fossils from the Mentone Caves. The skeletons of two children which had been buried together are in a splendid state of preservation, exhibiting admirably the characteristics of prehistoric cave-life. These two young people were buried in the home of their parents, very probably because it was the only means of defending their bones against the teeth of ferocious hyænas and other large carnivorous animals which were disputing with man the empire of the future Gaul.

from *Nature*, **12**, 358; August 26, 1875.

protein synthesis (by about three times) when injected into *X. laevis* oocytes. A fractionation of the RNA according to molecular weight showed that it was the RNA between 5S and 18S that gave the biggest stimulation. But stimulation of protein synthesis is completely inadequate as proof that the injected RNA is being translated. Stimulation of endogenous protein synthesis cannot be ruled out as an explanation unless the protein for which the injected messenger codes is shown to be synthesised in the oocytes. The endogenous protein synthesis in the oocyte is higher than in some cell-free systems. In spite of this disadvantage van der Donk was able to demonstrate the appearance of several new protein bands on gel electrophoresis of proteins synthesised in oocytes injected with plant RNA. Nonetheless it could be argued that these bands were oocyte protein whose *de novo* synthesis was stimulated by the plant RNA. To identify them as plant proteins van der Donk has attempted to demonstrate their function in the inhibition of pollen tube growth. Protein can be isolated from styles after self-pollination which, when transferred to a style that has been cross-pollinated, will inhibit the growth of pollen tubes in that style. Therefore a solution of proteins synthesised in oocytes in the presence and absence of RNA isolated from self-pollinated styles was applied to styles that had been cross-pollinated. Only the proteins synthesised in the presence of the plant RNA were able to inhibit the growth of the pollen tube. Van der Donk takes this as a clear demonstration that at least part of the proteins translated from plant RNA are those involved in the incompatibility reaction (the inhibition of pollen tube growth), and that by partially isolating the RNA coding for these proteins a further step has been made in the elucidation of the intriguing mechanism by which a plant distinguishes between self and non-self.

Gurdon and his colleagues have demonstrated the very long life—up to 10 days—of globin mRNA after injection into oocytes (Gurdon, Lingrel and Marbaix, *J. molec. Biol.*, **80**, 539; 1973). Allende and coworkers reasoned that this must be because oocytes contained a very low level of ribonuclease activity or else the RNA was protected in some way from degradation (Allende, Allende and Firtel, *Cell*, **2**, 189; 1974). To distinguish between these two possibilities they injected a variety of radioactive natural and synthetic RNAs into oocytes and followed their degradation with time. They were able to show that some RNAs were degraded and others were not, and concluded that the oocytes contained enough ribonuclease activity to degrade

injected RNAs so that those remaining must be protected in some way. They next focused their attention on yeast tRNA—one of the species that survived undegraded in oocytes even after 20 h. In this issue of *Nature* they report their demonstration that oocytes are capable of aminoacylating yeast tRNAs injected into cells even at final concentrations which were much greater than that of their endogenous tRNA content (page 675). They showed that oocytes can be injected with tRNA for phenylalanine to a final concentration in the cell 500 times the normal concentration—acylation taking place so that there is 150 times as much Phe-tRNA as normal. The authors plan to study the effects of this imbalance on the mechanism of protein synthesis.

Nuclear spectroscopy

from P. E. Hodgson

IN a nuclear transfer reaction angular momentum is conserved vectorially, so that the spin of the final state of the residual nucleus $J_R = J_T + j$, where J_T is the spin of the ground state of the initial (or target) nucleus and j the angular momentum of the transferred nucleon. This j is itself composed of its orbital and spin angular momenta, $j = l + \frac{1}{2}$. The angular distribution of the outgoing particle, for example that of a proton in a (d,p) reaction, is usually characteristic of the orbital angular momentum transfer l, and thus l can be determined. Since J_T is known, this suffices to set limits on J_R and to determine it in some special cases.

For example, if $J_T = 0$, $J_R = j = l + \frac{1}{2}$, so that $J_R = l \pm \frac{1}{2}$. So if $l = 2$, the final state is $d_{3/2}$ or $d_{5/2}$. This ambiguity may be resolved either by rather difficult measurements of the polarisation of the outgoing particle, or by careful examination of some special features of the differential cross sections (*Nature*, **250**, 464; 1974; **253**, 501; 1975).

The situation is more complicated if J_T is greater than zero, for then several values of j may be possible in reactions to states of the same J_R. Thus if $J_T = \frac{1}{2}$, $J_R = 1$, and $l = 1$, then j can be $\frac{1}{2}$ or 3/2. It is an interesting problem to determine j, for if this can be done we can learn more about the reaction and also use such reactions to determine J_R in cases where it is not known. Furthermore, j has to be known in order to apply the j-dependent sum rules to determine nuclear spectroscopic factors (*Nature*, **249**, 695; 1974).

Some years ago Kocher and Haeberli (*Phys. Rev. Lett.*, **23**, 315; 1969) showed that the vector analysing power in one-nucleon transfer reactions is

sensitive to the value of j and thus provides a good way of determining it. They obtained the vector analysing power from the left-right asymmetry of the protons emitted from a reaction initiated by a polarised beam of particles. The $j=\frac{1}{2}^-$ reactions ^{52}Cr(d,p)^{53}Cr ($Q=5.17$ MeV) and ^{54}Fe(d,p)^{55}Fe ($Q=6.60$ MeV) at 10 MeV had vector analysing powers quite different from the $j=3/2^-$ reactions ^{52}Cr(d,p)^{53}Cr ($Q=5.73$ MeV) and ^{54}Fe(d,p)^{55}Fe ($Q=7.01$ MeV) at 10 MeV. In the mixed-j reaction ^{53}Cr(d,p)^{54}Cr ($Q=6.71$ MeV) at 10 MeV, both these values of j were possible, and the vector analysing power closely followed a curve obtained by adding together in definite proportions the vector analysing powers found in the other two reactions. This proportion gave the relative contributions of the $j=\frac{1}{2}$ and $j=3/2$ components to the reaction. The technique of finding the mixing parameter in mixed-j transitions has the special advantage of being independent of detailed theoretical calculations, but is somewhat laborious to apply in practice.

A new method of finding the j value has recently been proposed by Dohan and Summers-Gill (*Nuclear Phys.*, **A241**, 61; 1975), and applied to the ^{69}Gd(d,p)^{70}Gd and ^{71}Gd(d,t)^{70}Gd reactions to the same state of ^{70}Gd. It depends on the fact that, on the simple shell model, the $2p_{1/2}$ neutron state is empty in ^{69}Gd, half-full (containing one neutron) in ^{70}Gd and full in ^{71}Gd. Thus the (d,p) and (d,t) reactions on ^{69}Gd and ^{71}Gd respectively go equally well, and thus have very similar spectroscopic factors. The $2p_{3/2}$ state, on the other hand, is full for all these nuclei, so the (d,p) reaction on ^{69}Gd is forbidden, as there is no room for another neutron, while the (d,t) reaction on ^{71}Gd takes place easily as there are plenty of $2p_{3/2}$ neutrons to be removed. This picture is expected to be somewhat blurred as the simple shell model is not followed exactly, but nevertheless we expect to find very similar amplitudes for $2p_{1/2}$ transfer and very different amplitudes for $2p_{3/2}$ transfer in the two reactions.

In practice these expectations are remarkably well fulfilled. The transitions to the lower states up to about 1 MeV have almost the same amplitudes for the two types of reaction while those above 1 MeV have very small amplitudes for the (d,p) reaction and large amplitudes for the (d,t) reaction. Thus we can with some confidence assign the first group to $2p_{1/2}$ transfer and the second group to $2p_{3/2}$ transfer. This is a very simple and direct way of determining the j value of the transferred particle, and is applicable to reactions where the sub-shell corresponding to one j value is filling more rapidly than the other. It

will be interesting to see how far it can be extended.

In heavy ion reactions the situation is more complicated since the transferred particle can have an angular momentum greater than zero in the projectile. Thus the neutron in the deuteron has $l=0$, whereas the proton in ^{7}Li that is transferred by the (^{7}Li,^{6}He) reaction has $l=1$, $j=3/2$ as it comes from an $p_{3/2}$ orbit in ^{7}Li (*Nature*, **253**, 501; 1975).

Sticky actin

from Dennis Bray

MUSCLE biochemistry is not usually the place to look for the bizarre; but what else could one call an association between muscle actin and an enzyme that digests DNA? The story is a curious one. As long ago as 1943, it was found that pigeon thymus contains a protein that inhibits deoxyribonuclease I—the abundant endonuclease of pancreas. This activity was found in other tissues but little else was learnt about its biochemistry until the late 60s when Lindberg—at the Karolinska Institute, Stockholm—began a careful study. He found that the inhibitor had a molecular weight close to 42,000; that it could be purified and even crystallised by conventional procedures; and that it formed a tight 1 : 1 complex with the nuclease. Among its notable characteristics, at least in retrospect, was its abundance in tissue (5–10% of the protein in a soluble extract), and its tendency to form aggregates of high molecular weight.

Then last year, at Cold Spring Harbor, New York, Lazarides and others interested in cell motility heard at first-hand of the DNase inhibitor. They also had a protein with molecular weight close to 42,000 which was strangely abundant in cells: only they called it actin. The amino acid compositions of the two proteins were encouragingly similar and the unlikely possibility was put to the test. Sure enough, the fingerprints of the two are the same, they cross react immunologically, and analytical-grade muscle actin sticks so tightly to DNase I that denaturing agents are needed to part them (Lazarides and Lindberg, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4742; 1974).

The interaction is unquestionably real but why does it occur? It is unlikely to be accidental because the binding is so tight. It may have some purpose, but it links two cellular processes that are normally considered to be independent. The puzzle is compounded by indications that actin forms other unexpected associations. Before the work mentioned above, Laki and Muszbek (*Biochim. biophys. Acta.*,

371, 519, 1974), had shown that actin in its filamentous form binds strongly to fibrin—the major structural protein of blood clots. And a recent paper examines the long known association between glycolytic enzymes, such as aldolase and triose phosphate dehydrogenase, and filamentous actin (Clark and Masters, *Biochim. biophys. Acta.*, **381**, 37; 1975). Still only in abstract form are claims that an interaction exists between actin and spectrin, the major protein of red blood cell ghosts (Tilney, *J. Cell Biol.*, **63**, 349a; 1974); and another report that actin might be identical to the γ-subunit of dogfish muscle phosphorylase (Fischer *et al*, *Hoppe-Seylers Z. Sür Physiol. Chem.*, **356**, 381; 1975)

If we consider the proteins that associate with thin filaments in muscle —troponin, tropomyosin, myosin and actinin—then we have ten or so that all bind to actin. The list might well be longer, for any club that includes both deoxyribonuclease and myosin must surely have a wide membership. It begins to look as though we are in the presence of a Phenomenon; but what it might mean is hard to say. Either actin is generally sticky and able to adhere to proteins it does not normally encounter, or many proteins have evolved binding sites that hitch them to this abundant and phylogenetically stable component of the cell

Evolution of *E. coli* chromosome

from Millicent Masters

IT has been possible to observe the intact chromosome of about half-a-dozen species of bacteria. In each species the DNA was found to be a closed circle. The circular chromosome of the best studied species of bacterium, *Escherichia coli*, contains enough DNA to code for about 3,000 average-sized polypeptide chains. Specific functions have been assigned to about 500 of these genes and the well developed genetics of *E. coli* has permitted their positions on the chromosome to be determined. Genes specifying proteins with related functions such as isoenzymes catalysing the same reaction or groups of enzymes involved in sequential metabolic conversions may be either close together or distant on the circular chromosome. Some related genes are close together because they are transcribed together (that is, they form an operon). Other related genes however are close together without sharing such a common control mechanism. Still other related genes, such as the five genes concerned with pyrimidine synthesis or seven of the 11 genes responsible for arginine biosyn-

thesis, occupy widely disparate locations on the chromosome.

David Zipkas and Monica Riley in a recently published article (*Proc. natn. Acad. Sci. U.S.A.*, **72**, 1354; 1975) have examined the locations of 125 genes, each of which was judged to be biochemically related to at least one other gene among the 125. With the help of a computer they analysed the distances between all pairs of genes and between all those pairs judged to specify biochemically related functions. They found that pairs of related genes were not placed randomly relative to one another but, rather, that they were almost twice as likely to be located 90° or 180° apart than might be expected by chance. To explain this remarkable observation the authors propose that the chromosome of *E. coli* is the product of two successive duplications of a primitive genome which was one quarter the size of the present-day chromosome. This event, postulated of course to have occurred a very long time ago, would have resulted in a tetrameric structure with each gene present four times at 0°, 90°, 180° and 270° positions.

In support of this idea they cite Wallace and Morowitz (*Chromosoma*, **40**, 121; 1973). These authors tabulated the genome sizes of about 70 species of bacterium and found that the sizes, which vary from about 0.5 to 3.0×10^9 daltons, do not vary continuously. *Mycoplasma* species have chromosome sizes of either 0.5×10^9 or 1.0×10^9 daltons. The other species recorded have genome sizes clustered around peaks at 1.4×10^9 and 2.8×10^9 daltons. These provocative data are consistent with the idea that smaller genomes have given rise to larger ones by a process of genome duplication.

Zipkas and Riley further propose that the dimeric or tetrameric products of genome duplication evolved to their present forms by mutation of, and divergence in function among, the duplicate gene copies. For example, duplicate copies of an ancestral gene, concerned with tryptophan metabolism could have evolved differently to yield in one case the gene for the tryptophan synthetase B protein and, 180° distant from it, the gene for tryptophanase. Both of these proteins can catalyse the conversion of indole and serine into tryptophan. Other cases however are more difficult to explain. The genes *arg* F and *arg* I which are neither 90° nor 180° apart both specify an ornithine transcarbamylase. Zipkas and Riley suggest that, although they specify proteins that carry out the same reaction, these two genes may have had independent origins (and as a consequence might be expected to have different sequences). Recent work, however, by Kikuchi and Gorini (reported at the

Fifth International Conference on the Biology of Temperate Bacteriophage, 1975) showing that the *arg* I and *arg* F genes have similar base sequences is inconsistent with this idea, and suggests that one of the genes arose by duplication of the other. Thus Zipkas's and Riley's suggestion that functionally related genes not 90° or 180° apart evolved independently is proved invalid in the one case where information on sequence homology is available.

It will obviously be difficult to either prove or invalidate the theory of Zipkas and Riley concerning the origin of the *E. coli* chromosome. Further information regarding sequence homology to support the necessarily somewhat arbitrary assignment of functional relationships will certainly be necessary. None the less, whatever the outcome of such studies, their provocative observation will remain to be explained.

Mitochondria and chloroplasts

from John Ellis

A pre-FEBS symposium on "The Structure, Synthesis and Functions of Nucleic Acids in Organelles" was held in Paris on July 19.

WITHIN the next year the complete genetic map of yeast mitochondrial DNA should be known. This bold prediction was made by P. Borst (Amsterdam University) whose optimism was generated by two recent technical advances. The first is the availability of restriction endonucleases, bacterial enzymes which cleave double-stranded DNA at specific base sequences into defined fragments which can be separated by gel electrophoresis. Hybridisation of the fragments with mitochondrial ribosomal RNA has revealed that the two ribosomal cistrons in yeast are not linked as they are in bacteria, but are located on opposite sides of the circular mitochondrial genome. This is not a constant feature of the mitochondrial genome however, since I. B. Dawid (Carnegie Institution, Baltimore) showed that the two cistrons are separated by a spacer region of only 120 base pairs in the mitochondrial genome of *Xenopus*, HeLa, and *Drosophila*.

The second advance is the production of a new class of mutations in the mitochondrial genome. P. Slonimiski (Gif-sur-Yvette) pointed out that, with the exception of mutations that confer resistance to antibiotics, most mutations in the yeast mitochondrial genome were of the so-called *rho* minus type where

large deletions of the mitochondrial DNA have occurred. In such mutants the protein-synthetic system of the mitochondria is not functional, and so no genetic mapping is possible. What is needed is a class of point mutations where proteins continue to be made but which lack specific enzymic activities. Recombination between such structural gene mutations should restore wild-type mitochondria and hence permit genetic mapping. Slonimiski reported the isolation of 120 such *mit* minus mutants; some confer normal cytochrome spectra but have no cytochrome activity, whereas others give abnormal spectra. Mapping to a resolution of 30–50 base pairs is in progress. It would be a mistake to think that there is a universal mitochondrial map however; Dawid pointed out that the mitochondrial DNA of sheep and goats differs by 80% in base sequence, thus lending substance to an old parable.

Restriction enzymes have also been used to study the DNA of the kinetoplast, an organelle related to the mitochondrion, and characteristic of trypanosomes and related protozoans. Each kinetoplast contains many thousand DNA minicircles of contour length 0 45 µm, as well as longer linear molecules. G. Riou (Gustave-Roussy Institute, Villejuif) reported kinetic complexity measurements on restriction fragments of this DNA; the minicircles show about 2% heterogeneity in base sequence, while the linear molecules contain a sequence of kinetic complexity similar to that found in mitochondrial DNA. No genes have yet been located in the kinetoplast genome, nor is the significance of multiple minicircles understood.

Higher plant and yeast mitochondrial DNA has a contour length and a kinetic complexity of 25–30 µm, while animal mitochondrial DNA is only 5 µm in length. Why should more genes be needed in plant mitochondria than in animal mitochondria? Borst suggested that 5 µm is sufficient for the structural genes required for all mitochondria, and that any extra length contained regulatory sequences. Slonimiski amplified this notion by pointing out that lengths longer than 5 µm were found either in facultative aerobes such as yeast, which might need more control sequences than obligate aerobes such as animals, or in green plant cells where the mitochondrion interacts metabolically with the chloroplast.

Chloroplasts from several higher plants and algae contain DNA circles of contour length about 45 µm: this has been regarded as a universal size for the chloroplast genome but R. Herrmann (University of Dusseldorf) reported that in the liverwort *Sphaerocarpus* the size was nearer 37 µm. Much less information is available about the function

of chloroplast DNA than about that of mitochondrial DNA. This is surprising in view of the primary role of chloroplasts in maintaining all living organisms. The view was expressed that this situation stems from the failure of too many departments of botany and biochemistry to promote interest in the biochemistry of plants; consequently far fewer biochemists study chloroplast development than study mitochondrial development. Several groups are currently preparing restriction fragments of chloroplast DNA, so some progress in mapping the chloroplast genome can be expected. One structural gene firmly established as located in this genome is that for the large subunit of Fraction I protein (ribulose–*bis* phosphate carboxylase). R. J. Ellis (University of Warwick) reported that this subunit was made *in vitro* by the free ribosomes of the chloroplast but not by the membrane-bound ribosomes. Isolated spinach chloroplasts will use light energy to incorporate ^{3}H-uridine into a discrete RNA species of molecular weight 2.7×10^6; M. R. Hartley (University of Warwick) and H. J. Bonert (University of Dusseldorf) reported hybridisation data which showed that this RNA species contains cistrons for both 16S and 23S chloroplast ribosomal RNA.

It is clear that most of the proteins of both mitochondria and chloroplasts are made on cytoplasmic ribosomes, and are then imported across the bounding membranes into the developing organelle. G. Schatz (University of Basle) suggested that the mechanism of specific protein uptake into these organelles is a major problem in cell biology, but no new data were presented at the meeting that shed any light on the process.

More and more promising

from David J. Miller

The International Particle Physics Conference was held in Palermo on July 23–28, 1975.

AT last year's International Particle Physics conference in London, some theorists placed bets that "charm" would be discovered before this year's conference in Palermo. Charm is a suggested new quantum number, like the well-established "strangeness" number carried by hyperons and K-mesons. It had been incorporated into a very plausible scheme to explain the weak interactions, including the recently established neutral current effects, but no one had seen a "charmed" particle.

Now the Palermo conference has passed, but it is still not clear who has won the bets.

Remarkable things have been discovered during the year, the psi (or J) and psi' particles in particular. In the last few weeks a collaboration working at the DORIS electron–positron colliding-beam machine near Hamburg has reported a new intermediate state between the psi and the psi'. They claim that when their e^+e^- collision energy was 3.7 GeV (that is, when they were making psi') they saw a number of events whose final state particles consisted of two fixed energy gamma rays and a psi. The psi was recognised when it delayed to a muon- or electron-pair with a mass of 3.1 GeV. The production of the two gamma rays is interpreted as a two-stage de-excitation cascade (psi'→new particle + gamma ray, followed by new particle→psi + gamma ray). Such cascades are encountered frequently in atomic or nuclear spectroscopy. Rumours from the SPEAR storage rings at Stanford, California suggest that similar intermediate levels have been observed there.

Charm lovers know exactly what they want these new objects to be. They say that the intermediate particle(s) are the p-wave states of "charmonium", that is, states with one unit of angular excitation. The psi is supposed to be an s-wave ground state, and the psi' is the first radial excitation. The states have been called charmonium by analogy with "positronium", a short lived atom-like structure formed from an electron and a positron. Charmonium should be made in a similar way from a charmed quark and a charmed antiquark. The only doubts one can have about this argument are that no one has yet proved that charm exists, and we haven't seen any quarks.

But progress has been made on both of these problems. The Stanford–Berkeley collaboration at SPEAR has now had time to collect e^+e^- annihilation data up to about twice the energy at which the psi' is produced. They have seen one other bump, wider than the psi and psi', centred at about 4.15 GeV, but they have also made a high-statistics run at a fixed rate collision energy of 4.8 GeV. This energy was chosen just because there seemed to be no specially interesting energy-dependent behaviour there. In the final states of this sample they have found evidence for another new phenomenon, the correlated production of muons and electrons. Combinations of a positive muon with a negative electron or of a negative muon with a positive electron have both been recognised, but there are no signs of combinations with the same charge for both the muon and the electron. The measured momenta

of the electrons and muons show that they do not carry 4.8 GeV of energy between them, so neutral particles must have escaped undetected.

Charm lovers are, once more, ready with an explanation. A pair of charmed mesons (real charmed particles, not charmonium) has been produced, they say. They must carry opposite charges, and they decay just like pions or K-mesons to muons or electrons, with neutrinos and perhaps neutral pions to take away the unseen energy. It is interesting that a very similar decay process for charmed particles could explain the neutrino-induced events with pairs of muons in the final state, reported at last year's conference by the Harvard–Pennsylvania–Wisconsin–Fermilab group, and confirmed this summer by the Caltech group, also working at Fermilab near Chicago. It is beginning to look as if the charm lovers may be right, but none of the evidence is really firm yet and physicists like to be sure before they pay out on a bet.

As for the problem of seeing quarks, theoretical physicists now tell us that we should not even try. Quarks exist, they say, but they can never be seen. They explain many of the properties of all strongly-interacting particles. They carry most of the mass, as well as the charge, strangeness and charm quantum numbers, but they can never be shaken free from the groupings of three quarks, or quark and antiquark, out of which particles are built. A group at the Massachusetts Institute of Technology has invented a model in which the quarks are permanently confined in "bags" about 10^{-15} m across. Other groups, including one at Cornell University, have models in which the quarks are tied on strings. Until recently these models were regarded as purely phenomenological; just rigged up to fit the data. But at Palermo, and afterwards at the Erice summer-school, near Palermo, it has become clear that fundamental field theories of elementary particles might provide very natural and attractive schemes in which quark-confinement could be achieved. This would be very exciting, since it could lead to a unified theory in which the strong, weak and electromagnetic interactions could be explained at the same time. At the moment some of the leaders in this effort, such as Coleman from Harvard or 't Hooft from Utrecht, are still proving beautiful theorems about two-dimensional space–time. K. Wilson of Cornell works in four dimensions, but his space and time form a quantised lattice. It may take a few years to get to the four-dimensional space–time continuum that physics actually happens in, but many theorists are very optimistic; although nobody is placing any bets.

review article

Did Chinese cosmology anticipate relativity?

John Gribbin*

Recent interest in historic novae and supernovae, stimulated by the discovery of pulsars, has shown the value of ancient Chinese astronomical observations. But the Chinese also have a long record of "cosmological" theorising; in some cases their old concepts regarding the Universe are remarkably similar to those developed in the West during the twentieth century.

ANY discussion of astronomy and cosmology in China in historic times must inevitably draw on Needham's work[1]; it is therefore appropriate to begin such a discussion in what for an astronomer is the sobering light of the quotation from Franz Kühnert, made in Vienna in 1888 and which graces the title page of ref. 1: "Probably another reason why many Europeans consider the Chinese such barbarians is on account of the support they give to their Astronomers—people regarded by our cultivated Western mortals as completely useless. Yet there they rank with Heads of Departments and Secretaries of State. What frightful barbarism!"

Those "barbarians" were, however, thinking about the nature of the Universe, and producing some remarkably modern concepts, at least two thousand years ago. The earliest concept, of the heavens as a hemispherical dome closing over the Earth, and the slightly later concept of a celestial sphere surrounding the spherical Earth are familiar enough; it is possible that the earliest ideas can be traced back to Babylonian concepts of the world, and that the theories migrated both westward to Greece and eastward to China, where they developed independently. But it is particularly striking that one school of thought does not seem to have displaced the other in China. Followers of either theory could be found from the fourth century BC onwards, and there were also other schools. In terms of modern cosmology, the most striking of these ideas was not, it seems, the most popular some 2,000 years ago—but it was taught, and it is this system that most resembles modern ideas.

Infinite empty space

The Hsüan Yeh theory of the Universe was a later development and little is known about it before the Later Han (AD 25–AD 220). A little later, Ko Hung wrote (see ref. 1 p. 219): "The books of the Hsüan Yeh school were all lost, but Chi Mêng, one of the librarians, remembered what its masters before his time had taught concerning it. They said that the heavens were empty and void of substance . . .

"The sun the moon and the company of stars float freely in the empty space, moving or standing still. All are condensed vapour."

And in AD 336 Yü Hsi, the Chinese discoverer of the precession of the equinoxes, wrote: "I think that the heavens are

*Address: *Nature*, 4 Little Essex Street, London WC2R 3LF, UK.

infinitely high, and that the space below the earth is unfathomably deep."

These ideas were some 1,300 years ahead of western thought (as far as there was any western thought about the nature of the Universe in the fourth century), and it is particularly interesting that western cosmology only broke the bounds imposed by the concept of the "crystal spheres" at about the time that reliable news of Chinese philosophy, mathematics and astronomy was beginning to reach the West. Not that the new ideas were broadcast as a great revelation by the Jesuits who visited China at the end of the sixteenth and beginning of the seventeenth centuries: they were held up to ridicule as examples of the stupidity of Chinese philosophers. Matteo Ricci listed several "absurdities" in Chinese astronomical thought in letters written in 1595 (see ref. 1 p. 438) including: "They say that there is only one sky and not ten skies; that it is empty and not solid. The stars are supposed to move in the void, instead of being attached to the firmament . . . Where we say there is air between the spheres, they affirm there is a void".

But these "absurdities" found a receptive audience in some western thinkers, at least. This was the time when western astronomy began to develop as a science, rather than as a branch of religion, and this development has led, 300 years later, to the present picture of an infinite Universe dotted with island galaxies. It is ironic that just when astronomy was making the crucial break with religion, orthodox western religious views were imposing a barrier on the further development of Chinese astronomy. The Jesuits persistently failed to broadcast new ideas such as those of Copernicus in China, and the centre of development of the science had definitely shifted to the West by the end of the seventeenth century. Even more ironically, a further 300 years of development in the west has only, it seems, brought cosmology to a level reached in China well before the time of the Jesuit mission. Even in the thirteenth century the concept of the plurality of worlds was well established, and Têngu Mu wrote (see ref. 1 p. 221): "Heaven and Earth are large, yet in the whole of empty space they are but as a small grain of rice . . . Empty space is like a kingdom and Heaven and Earth no more than a single individual person in that kingdom. How unreasonable it would be to suppose that besides the Heaven and Earth we can see there are no other heavens and no other earths."

To make that a modern statement, it is necessary only to replace "heavens" by "galaxies"; and if that concept had reached western cosmologists such as Thomas Wright, who

published a remarkable theory of the structure of our Galaxy in 1750 (ref. 2), who knows what the consequences for the development of cosmology might have been.

Space and time

Perhaps the most important difference between the ancient Chinese thinkers and their western contemporaries was openness of mind. The Chinese were always, it seems, prepared to consider different theories of the Universe, rather than subscribing to

Fig. 1 Chinese oracle bone dating from about 1300 BC with the oldest existing written record of a nova (from Needham[1]).

rigid doctrine. They also had a rather better grasp of the size of the Universe, both in space and time. Buddhist ideas of a cyclical Universe may have helped with the development of ideas about time; certainly the eighth century Chinese estimate of the time elapsed since the last "conjunction" (96,961,740 yr; see ref. 3 p. 45) was, while perhaps 100 times too small by modern estimates, still a lot more realistic than a European bishop's notorious eighteenth century estimate that the Earth was created on a certain day in 4004 BC, at six o'clock in the evening.

This grasp of space and time extended even to more abstract ideas. In a text of 120 BC quoted by Needham (ref. 3 p. 219) we find the following definition: "All the time that has passed from antiquity until now is called chou; all the space in every

direction, above and below, is called yü." Thus, the Chinese expression yü–chou, commonly translated as "The Universe", means more literally "space–time"—a concept which only gained respectability in the West a few decades ago. This is not the only example of how the great philosophers of China seemed to foreshadow ideas now firmly attributed to Einstein. As early as 300 BC the Mohists taught (ref. 3 p. 221): "Movement in space requires duration . . . In movement, the motion of an observer must first be from what is nearer, and afterwards to what is further. The near and far constitute space. The earlier and later constitute duration. A person who moves in space requires duration." Although these ideas were so advanced, they have never, it seems, directly influenced the development of western ideas. That cannot be said of Chinese observational astronomy, however, which is even now providing vital fundamental information used by the most modern astronomers of all, the high energy astrophysicists.
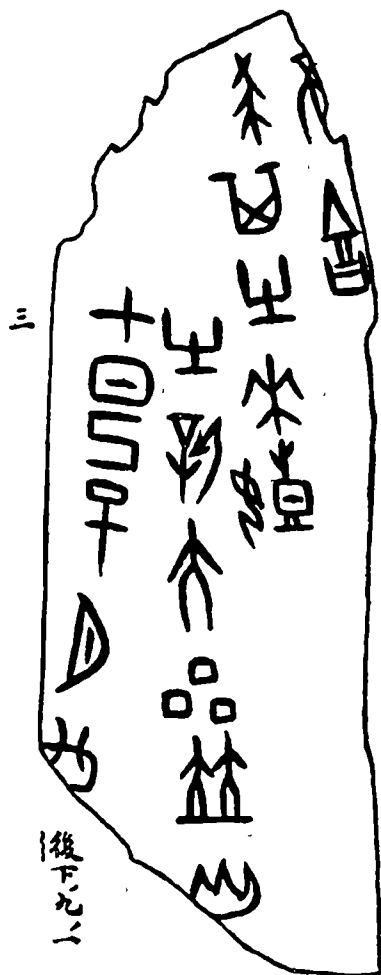
Guest stars and Uhuru

Although it was the discovery of pulsars which first stirred renewed interest in Chinese records of guest stars, the most fruitful development recently has stemmed from observations of X-ray stars, made from the satellite Uhuru. The importance of the Chinese observations is that they provide accurate dates for the supernova explosions in which, it is generally believed, pulsars and X-ray stars are born. These sources are evolving so rapidly that information about their ages, and thus of their development over the past 2,000 yr or so, is of great importance.

The most famous Chinese guest star is, of course, the event recorded in June of AD 1054, which gave birth to the Crab Nebula, with its associated pulsar and X-ray source. But Cowley and MacConnell have identified six other X-ray stars with six guest stars dating from AD 1690, AD 1203, AD 902, AD 827, AD 722 and 48 BC (ref. 4). Some of these identifications are tentative, because the Chinese records are not always as accurate as modern astronomers would like (see Fig. 1). But if they are correct, they also provide a new insight into those ancient observations. Assuming that the X-ray stars have not moved far since they were created in the supernova explosions, the combination of modern positional information and the old records provides an accurate calibration of the Chinese unit of angular measure, and this in turn makes other old Chinese records more valuable.

There is still further interaction between modern astronomy and the Chinese records. Kiang, for example, has calculated the orbit of Halley's comet over the millenia[5], and compared the calculated dates with dates of the appearance of comets (also referred to as guest stars) in the Chinese literature. That provides a new calibration of the Chinese calendars, and helps both historians and astronomers to make best use of the wealth of information available about China.

Perhaps there are still unsuspected ways in which this information might contribute to modern astronomy and cosmology; certainly the ideas are worthy of more than passing mention in teaching the subjects, and it would do no harm to students to draw their attention to such theories as the idea noted in a first century BC book (see ref. 1 p. 224): "The Earth is constantly in motion, never stopping, but men do not know it; they are like people sitting in a huge boat with the windows closed; the boat moves but those inside feel nothing."

[1] Needham, J., *Science and Civilisation in China*, 3 (Cambridge University Press, London, 1959).
[2] Wright, Thomas, *An Original Theory of the Universe* (Facsimile reprint of 1750 edition, Macdonal, London, 1971).
[3] Needham, J., *The Grand Titration* (Allen and Unwin, London, 1969).
[4] Cowley, A. P., and MacConnell, D. J., *Astrophys. Lett*, 11, 217 (1972).
[5] Kiang, T., *Mem. R. astr. Soc*, 76, no 2 (1972).

articles

Similarity of genes *argF* and *argI*

Akihiko Kikuchi & Luigi Gorini

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Two genes, at different positions on the Escherichia coli *genetic map but producing two enzymes which catalyse the same reaction, have a structure so similar that their DNAs anneal with each other in a heteroduplex mapping test.*

COMPARED with eukaryotes, bacteria rarely have multiple copies of a gene. In some cases the duplicated gene is located very close to the original copy, usually in tandem as in the case of certain transfer RNAs[1]. In other cases they are scattered without unique orientation, as in the case of the genes of rRNA, of which there are 5–7 copies[2]. A need to increase gene dosage may account for these situations. The apparent lack of such a requirement makes the case of ornithine transcarbamylase

(OTC) more intriguing. This enzyme of the arginine synthetic pathway which converts ornithine to citrulline, is coded for by gene *argI* in all strains of *Escherichia coli* and *Salmonella* and is located at 85 min on the *E. coli* linkage map. In *E. coli* strain K12, however, an additional gene copy for the same enzyme, gene *argF*, exists and is located at 7.5 min of the *E. coli* map[3]. Why strain K12 should possess two OTC genes is not clear. The products of each of these two genes, *argI* and *argF*, have been studied biochemically. It has been found that four types of a trimeric enzyme molecule are formed by the random combination of two slightly different subunits coded for by each gene. The difference of each gene product is detected by ion exchange chromatography but not by size and shape of the subunits[4]. In addition the trimeric molecule formed by the *argI* product, is considerably more heat resistant than that consisting of the *argF* product (Glansdorff, personal com-

Fig. 1 Heteroduplex structure of λ specialised transducing phages. Heteroduplexes of DNA between λ*c1857 S7* phage and each λ transducing phage are represented in the linear drawings. The double strand regions are represented proportionally while single strand loops are not. The double strand portion was measured by a map-measurer from a suitable enlargement and average values obtained from 10–20 heteroduplex molecules were normalised using *b519*, *b515* deletion loops as internal markers[10]. Total λ*c1857 S7* phage DNA is 16.4 μm long whereas λ*y*199 (carrying the two deletions *b519*, *b515*) is 14.6 μm long using this type of measurement (error 5%). The distances indicated in the drawings are in μm. All material including bacterial and phage strains and methods has already been described[5] except for the heteroduplex mapping technique. After some failures encountered by using the recommended technique for the DNA of φ80 phage particles[8], we modified the technique for λ phage particles as follows: 1–5 μl of the λ phage (10^{10} ml^{-1}) in CsCl solution ($\rho = 1.5$ g cm^{-3}) is added to 5 μl of 0.4 M EDTA-Na$_3$ and left at room temperature for 10 min. The phage solution was then diluted to a final volume of 25 μl by double-distilled water and mixed with 25 μl of another phage solution processed in the same way. The mixture of the two phages (50 μl) is diluted to 400 μl by adding 10 mM Tris-HCl *p*H 8.8, then denatured by 10 μl of 5 N NaOH for 30 min at room temperature and then neutralised by 60 μl of fresh 1:1 mixture of 2 M Tris-HCl *p*H 7.4 and 1.8 M HCl. The renaturation was started by the addition of 0.47 ml of 99% formamide and continued for 36 h at room temperature. During the renaturation a 60 μl of Tris-HCl *p*H 8.8 was added to prevent the lowering of the *p*H.
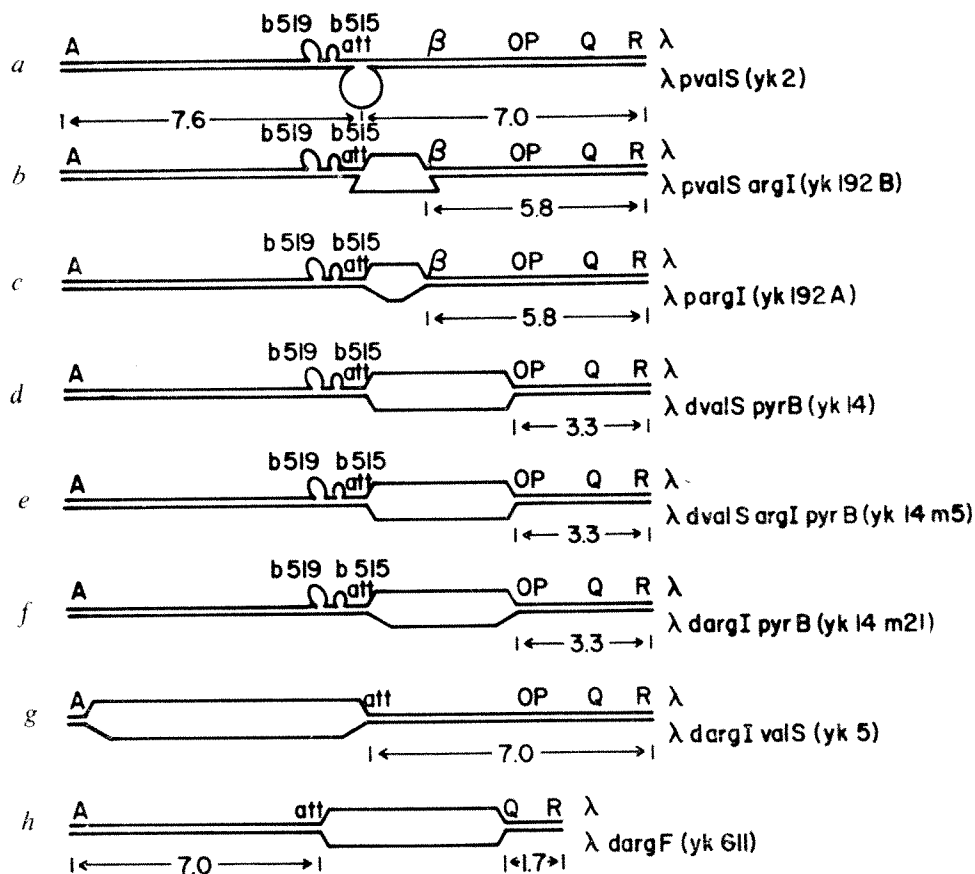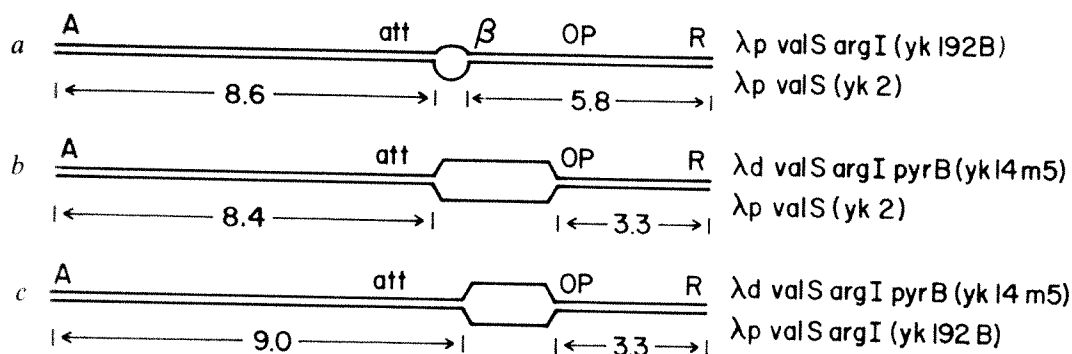
Fig. 2 Heteroduplex structure of λ specialised transducing phages carrying the *valS argI pyrB* region. The linear drawing represents heteroduplexes between each λ transducing phage. Details are described in the legend of Fig. 1.

munication). All other kinetic and affinity constants of the two enzymes are indistinguishable as well as the regulation pattern of the two operons, both being controlled to the same extent by the same repressor molecule, the *argR* product. Thus, the bulk of the two operons, *argF* and *argI*, which include promoters, operators and structural genes, might be so similar that the two DNA sequences could be able to anneal to a large extent if not completely.

Heteroduplex structure of the transducing phages

To demonstrate this hypothetical DNA homology, we first constructed several specialised transducing phages in the *argI* and *argF* regions of *E. coli* K12 chromosome. The structure of their heteroduplexes with the corresponding wild-type phages is presented in Fig. 1. In the *argI* region, because of two well defined markers, *pyrB* and *valS*, closely located at either side of the *argI* gene and 97% cotransducible with *argI* by phage P1, we could easily isolate λ specialised transducing phages of different sizes in that region of the chromosome. In addition to the previously isolated and described[5] phages λp*valS2* (yk2), λd*argI valS* (yk5) and λd*valS pyrB* (yk14), we have obtained the following phages. λp*valS argI* (yk192B) and λp*argI* (yk192A) were isolated from the HFT lysate obtained from strain RW420 (*proA/B argF lac*)ᵛ (*gal attλ uvrB*)ᵛ lysogenised with λp*valS2* (yk2) phage using the *valS* gene homology. LFT transductants Arg⁺ were selected using the strain AD1 (*proA/B argF lac*)ᵛ *argI*ᵛ as recipient. On heat induction they produced HFT lysates of two types of phages, λp*valS argI* (yk192B) and λp*argI* (yk192A). The lytic growth of yk192B always gives rise to a mixture of yk192B and yk192A (distinguishable by CsCl density gradient centrifugation) in different ratios depending on the conditions of the culture. The heteroduplex mapping shown in Fig. 1b and c, reveals that the λp*argI* phage arises from λp*valS argI* by losing the *valS* portion. In a similar way the phage λp*valS2* (Fig. 1a)

tends to lose *valS* and return to the parental phage λy199 during lytic infection.

Phages λd*valS argI pyrB* (yk14m5) and λd*argI pyrB* (yk14m21) (Fig. 1e and f) were selected from the lysate of strain BC22 *argI⁺ pyrB⁻*, lysogenised with phage λd*valS argI*ᵛ *pyrB⁺* (yk14). In this way rare recombinants with the host chromosome appear which are Arg⁺. As seen in Fig. 1, there is no visible increase in DNA size in yk14m5, while there is a definite decrease in yk14m21 when they are compared with yk14 (Fig. 1d). The DNA length lost is of the order of 1.5 μm, which corresponds to the size of *valS* insertion in λp*valS2* phage (Fig. 1a). In the *argF* chromosomal region we obtained the phage λd*argF* (yk611) (Fig. 1h) as already described[5].

Physical mapping of the *argI* region

To demonstrate the exact location of *argI* genes in the transducing phages, we made a heteroduplex analysis between transducing phages themselves. Figure 2 summarises the heteroduplex map of combinations of phages λp*valS*, λp*valS argI*, and λd*valS argI pyrB*. The gene order can be qualitatively recognised as *valS→ argI→ pyrB* starting from the attachment site, by the estimation of the length of double strand DNA and the size of single strand loop. For example, the double strand region at the left side of Figure 2a and b is the same and that at the right side of Fig. 2a is longer than that of Fig. 2b, indicating that *valS* gene is located at the left of *argI*. An analogous comparison between Fig. 2b and c permits the conclusion that *argI* is at the left side of *pyrB*. In addition, the short single strand loop in Fig. 2a should contain the *argI* gene, while the longer loop in Fig. 2b must contain *argI* and *pyrB* genes. The loop in Fig. 2c should contain at least the *pyrB* gene and probably something more at the right side. Figure 3 gives a clear view of the region because the heteroduplex between λp*argI* and λd*valS argI pyrB* phages directly shows the *valS* size as an insertion loop at the attach-

Fig. 3 Electron micrograph of heteroduplex DNA between λd*valS argI pyrB* (yk14m5) and λp*argI* (yk192A). A drawing is shown at the bottom in the same way described in Fig. 1. Black bars represent 1 μm. The specimen for electron microscopy was prepared and examined as described[5]. We did not expect any heteroduplex between *l* and *l* strands (or *r* and *r* strands) because if any homology exists in the homologous strands of two genes oriented in opposite directions this will be too small to stabilise such a heteroduplex unless each strand is separated and then mixed with the corresponding strand[9]. Therefore, if we did not see any homology among a number of heteroduplexes (*l* × *r*) we concluded that either there is no homology between the corresponding parts of the gene or that the two genes in question are oriented in opposite directions.
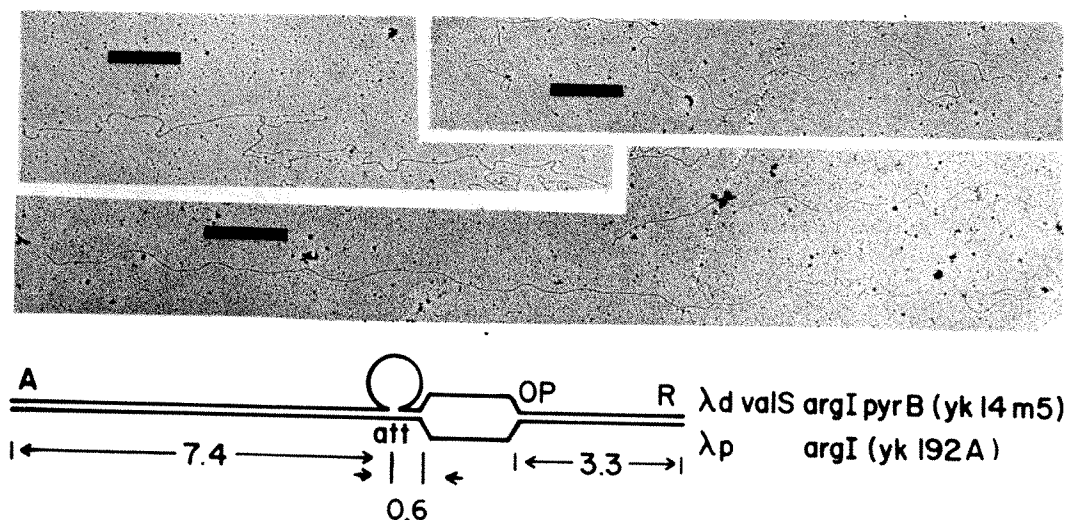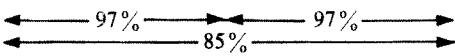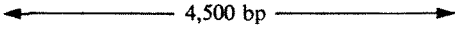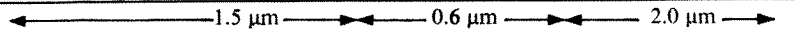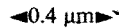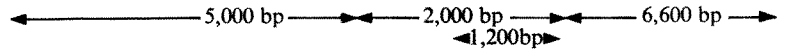
Table 1 The size and order of the *valS argI pyrB* gene

Genetic marker	— *valS* —	— *argI* —	— *pyrB* —
P1 cotransducing frequency	←——— 97% ———→←	——— 97% ———→	
	←——————— 85% ——————→		
DNA base pairs calculated from P1 transduction	←——————— 4,500 bp ———————→		
EM observation from Fig. 3	←——— 1.5 μm ———→←	— 0.6 μm —→←	—— 2.0 μm ——→
EM observation for *argF/argI* duplex from Fig. 4		◄0.4 μm►	
DNA base pairs calculated from EM	←——— 5,000 bp ———→←	— 2,000 bp —→←	—— 6,600 bp ——→
		◄1,200bp►	
Molecular weight of the protein	110,000	35,000	33,000+17,000
DNA base pairs expected from the molecular weight	3,300 bp	1,050 bp	1,000 bp+500 bp

The following conversion indexes are used: 1 μm DNA length = 3,300 base pairs; 0.1 min map distance = 4,500 bp; 1,000 bp correspond to a protein of molecular weight 33,000.

ment site, *argI* as a small successive double strand section and *pyrB* as a single stranded loop after *argI* at the right end.

The maximum size of each gene can be calculated as follows. The *valS* insertion is about 1.5 μm which corresponds to about 5,000 base pairs (bp), the *valS* gene itself requiring only 3,000 bp. The *argI* portion is 0.6 μm corresponding to 2,000 bp, but the *argI* gene itself should require only 1,000 bp. There is therefore a considerable space between *valS* and *argI* (about 1,000 bp at least) unaccounted for, which could indicate either another unknown gene or a mere spacer sequence. Finally *pyrB* has a length of 6,600 bp which is certainly too long for the *pyrB* gene. Since we do not have any marker after *pyrB* closer than *fdp* (which is separated by 15,000 bp calculated by P1 transduction frequency[6]), we could not obtain any phage for *pyrB* substitution enabling us to be more precise about the size of the *pyrB* gene. As it may be seen from Table 1, the overall distance of the three genes, *valS*, *argI* and *pyrB* is coincident with the P1 transducing frequency data.

argI, argF gene homology

From the physical mapping of the *argI* region we confirm the conclusion reached previously[5] about the orientation of the *argI* gene in the λ phage. Since it is known that the operator of *argI* is located between *valS* and *argI* (ref. 7), we conclude that the *argI* gene is transcribed from left to right in the phage λd*valS argI pyrB*. The same must be true for the phages λp*valS argI* and λp*argI* on the basis of the heteroduplex mapping results. By contrast, in the case of phage λp*argI valS* (yk5), the orientation of *argI* must be the opposite since we could not detect homology between *valS argI* of λp*valS argI* and *valS argI* of λd*argI valS*. If there is considerable homology detectable by electron microscopy between *argF* and *argI* then we should see the *argF–argI* duplex in the heteroduplex either between λd*argF* and λd*valS argI pyrB* or between λd*argF* and λd*argI valS* (yk5). If there is no homology at all between the two OTC genes, we would not obtain a duplex in either combination. Figure 4*a* and *b* shows that duplex *argF–argI* is obtained

a   A   Q R   λd valS argI pyrB (yk 14 m5)
|←——— 7.0 ———→| →| |← |←1.7→| λd argF (yk 611)
          0.4

b   A   Q R   λp argI (yk 192 A)
|←——— 7.0 ———→| →| |← |←1.7→| λd argF (yk 611)
          0.4

Fig. 4 Electron micrograph of heteroduplex DNA between λd*argF* and λd*valS argI pyrB* (*a*) or λp*argI* (*b*). Black bars represent 0.5 μm.

Table 2 Orientation of *E. coli* chromosomal markers on the λ transducing phage
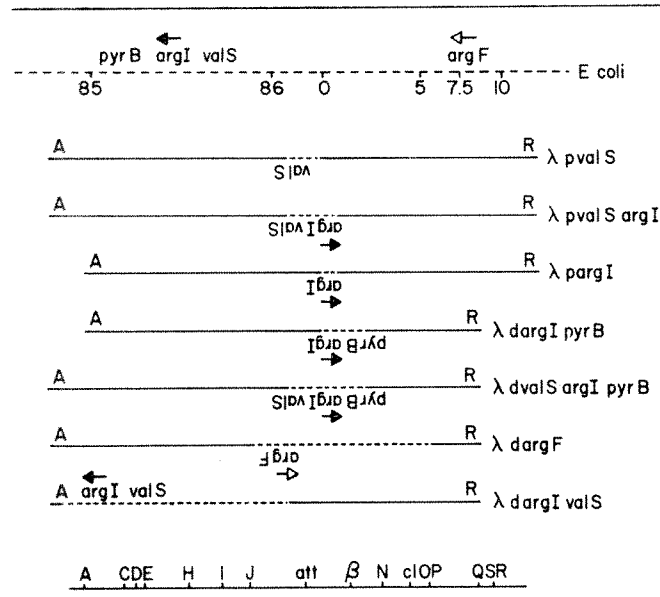
The phage genes are represented at the bottom of the figure by a straight line with A end at the left and R end at the right. The *E. coli* chromosome is shown as a broken line and relative places of genes are shown on the top. The letter orders of each genetic marker are kept as it is in Taylor's map[11] to demonstrate the way in which the chromosomal portion was inserted into the λ genome. The assignment of gene order of λ*pvalS*, λ*pvalS argI*, λ*pargI*, λ*dargI pyrB*, λ*dvalS argI pyrB* and λ*dargI valS* are described in the text. The arrow on the *argI* represents the transcription direction from the operator mappin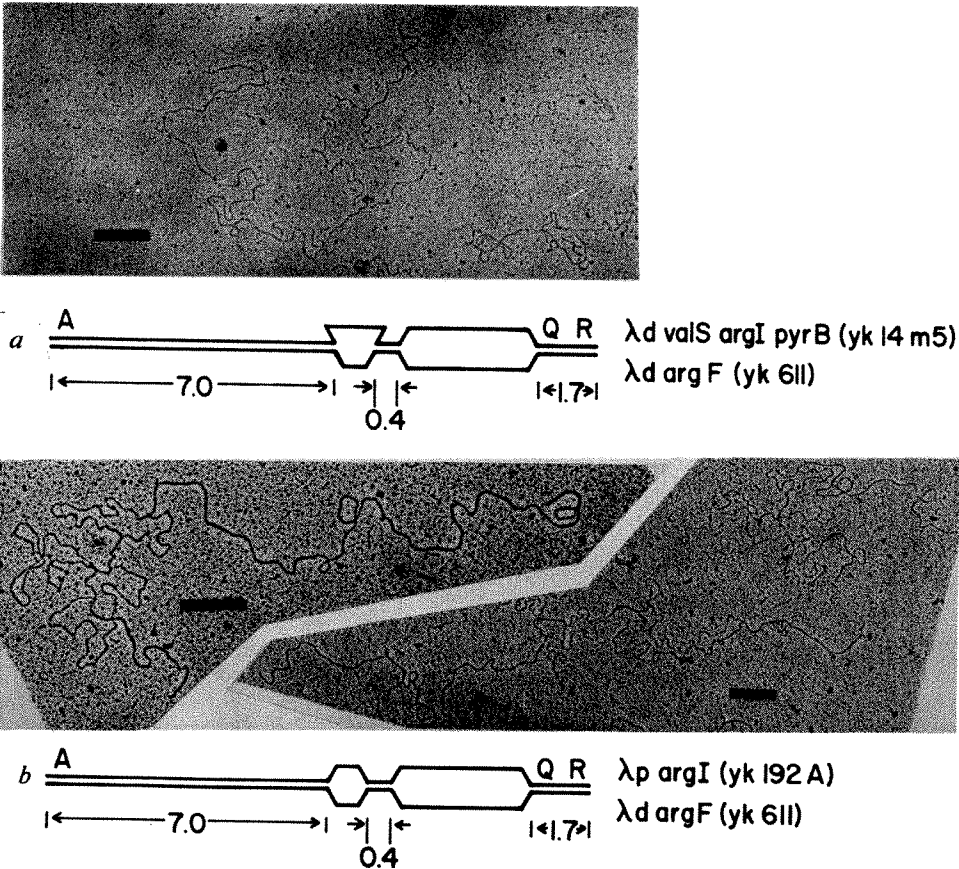g data of G. Jacoby[7]. The open arrow on the *argF* is determined from the fact that *argF* gene is annealed with the *argI* gene of λ*dvalS argI pyrB* phage.

with the combination λ*dargF* and λ*dvalS argI pyrB* or λ*pargI*. The location of duplex DNA between two single strand loops is exactly where *argI* gene is expected from the physical mapping data so that we believe that the duplex is between *argF* and *argI* although we could not confirm as yet whether the side opposite *argI* is indeed *argF* itself. The determined length of 0.4 μm (1,200 bp) is in agreement with the size of 1,050 bp for

an OTC subunit (molecular weight, 35,000). We could not observe any heterogeneity within this duplex region although some minor base difference should be expected between the two OTC genes. Also we could not demonstrate any evidence for or against the operator–promoter homology. The size of 1,200 bp could accommodate such a region.

This result shows that the DNA sequences of *argI* and *argF* are extensively similar. Moreover, since the annealing extends for about 0.4 μm which is the maximal length for the *argI* (or *argF*) gene, we can conclude that the homology extends over the entire length of the two genes. We do not expect that it should cover more because the surrounding genes at 85 and 7.5 min in the *E. coli* chromosome are completely different. Concerning orientation, our results only show that in this particular phage, yk611, the reading of *argF* is from left to right, the same as in phages λ*pvalS argI pyrB* or λ*pvalS argI* with which it anneals. The actual orientation of *argF* in the bacterial chromosome can only be assessed if we know in which way the *argF* has been inserted in the λ genome. According to considerations developed previously[5] this insertion in yk611 is inverted in respect to the bacterial chromosome. If this conclusion is correct, then the *argF* gene orientation in the bacterial chromosome is counterclockwise as it is for the *argI* gene. These results are summarised in Table 2.

1 Russell, R. L., Abelson, J. N., Landy, A., Gefter, M. L., Brenner, S., and Smith, J. D., *J. molec. Biol.*, **47**, 1–13 (1970).
2 Deonier, R. C., Ohtsubo, E., Lee, H. J., and Davidson, N., *J. molec. Biol.*, **89**, 619–629 (1974).
3 Glansdorff, N., Sand, G., and Verhoet, C., *Mutat. Res.*, **4**, 743–751 (1967).
4 Legrain, C., Halleux, P., Stalon, V., and Glansdorff, N., *Eur. J. Biochem.*, **27**, 93–102 (1972).
5 Kikuchi, A., Elseviers, D., and Gorini, L., *J. Bact.*, **122**, 727–742 (1975).
6 Fraenkel, D. G., *J. Bact.*, **93**, 1582–1587 (1967).
7 Jacoby, G. A., *J. Bact.*, **108**, 645–651 (1971).
8 Ohtsubo, E., Lee, H. J., Deonier, R. C., and Davidson, N., *J. molec. Biol.*, **89**, 599–618 (1974).
9 Shapiro, J., MacHattie, L., Eron, L., Ihler, G., Ippen, K., and Beckwith, J., *Nature*, **224**, 768–774 (1969).
10 Szybalski, E. H., and Szybalski, W., *Biochimie*, **56**, 1497–1503 (1974).
11 Taylor, A. L., and Trotter, C. D., *Bact. Rev.*, **36**, 504–524 (1972).

Nucleotide sequence of a viral RNA fragment that binds to eukaryotic ribosomes

Ranjit Dasgupta, Ding S. Shih, Chris Saris & Paul Kaesberg

Biophysics Laboratory of the Graduate School, and Biochemistry Department of the College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706

The nucleotide sequence has been determined for the first 53 bases of brome mosaic virus RNA4, the monocistronic messenger for brome mosaic virus coat protein. The sequence includes the binding site for wheat embryo ribosomes. The 5'-terminal base is a modified guanosine attached to the penultimate base through a 5' p-p-p 5' link. The initiating AUG codon is only 10 nucleotides from the 5'-terminus. The 13 triplets following the AUG codon correspond to the known sequence of brome mosaic virus coat protein.

IN the past few years, studies of nucleotide sequences of bacteriophage RNAs[1-8] have provided valuable information regarding the structure of initiation sites for protein synthesis in prokaryotes, but how ribosomes are able to select such initiator regions is still an open question. No sequences are known for the binding site of any eukaryotic RNA although recent progress with *in vitro* translating systems[9] utilising such messengers has made sequence investigations both feasible and interesting. We report here the ribosome binding site of brome mosaic virus (BMV) RNA4 (Russian strain). BMV RNA4 is the smallest of the 4 RNAs contained in the virions of BMV, a multicomponent plant virus[10,11] infectious to wheat. BMV RNA4 has been

shown to be an efficient, monocistronic messenger for BMV coat protein synthesis in a cell-free protein synthesising system derived from wheat embryo[12].

Isolation of fragments containing ribosome binding site

A number of workers[4-8] have isolated ribosomal binding sites of bacteriophage RNAs, taking advantage of the fact that regions of RNA can be protected from nuclease digestion by their association with ribosomes. When we tried to isolate a ribosome–mRNA complex in this way with BMV RNA4 and wheat embryo ribosomes, we obtained a series of ribosome-protected fragments which appeared on polyacrylamide gels as closely stacked bands. Fingerprint analyses showed that these fragments contain initiation sequences, but it proved difficult to purify any one fragment in an amount sufficient for detailed analysis.

Rather than using ribosomes to protect binding sites from nuclease digestion, we found it preferable to use ribosomes to select mRNA fragments that contain binding sites. Wheat embryo ribosomes will bind certain BMV RNA4 fragments selectively. Among them are fragments that contain the codons for the N-terminal region of BMV coat protein, and these could be obtained in relatively pure form by choosing appropriate fractions from polyacrylamide gels.

The selection and purification of the binding site fragment were as follows. ^{32}P-labelled BMV RNA4 was digested under very mild conditions with ribonuclease T_1 and a part of the digest was subjected to polyacrylamide gel electrophoresis to examine the extent of degradation. The gel pattern is quite reproducible, indicating the introduction of cleavages at specific sites of the RNA. It contains one major band and many minor bands corresponding to fragments of various lengths. (Analysis of the major band showed that it is a fragment about 150 nucleotides long. It comes from the 3′-end of the RNA molecule and does not contain the ribosome binding site. Its characteristics will be reported elsewhere.) For ribosome binding, the partial digest was added to wheat embryo extracts under conditions that allow initiation of protein synthesis. Elongation of nascent peptide chains was inhibited by the addition of the antibiotic anisomycin[13]. Following incubation, the mixture of ribosomes and RNA fragments was centrifuged through a sucrose density gradient (Fig. 1). About 5% of the total radioactivity sedimented in the region of 80S, indicating a binding of some of the fragments to 80S ribosomes. Fractions containing the ribosome–fragments complex were combined and were extracted with phenol. RNA, consisting of unlabelled rRNA and radioactive RNA fragments, was precipitated from the aqueous layer with ethanol. The resuspended RNA was heated in 6 M urea at 60 °C, followed by a sucrose gradient sedimentation in the presence of urea. All the radioactive RNA was thus dissociated from the bulk of the rRNA and remained near the top of the gradient. Fractions containing radioactivity were pooled and the RNA was precipitated with ethanol. Figure 2a shows the gel electrophoresis pattern of this RNA. Its appearance is different from that of the unbound digest both in distribution and absolute amount of radioactivity. The pattern of Fig. 2a consists of two bands (bands I and II), corresponding to RNA fragments of relatively small size, along with bands corresponding to larger fragments of a variety of lengths. Sequence analysis (see below) showed that the fragments of bands I and II were indeed ribosome binding sites, and the most prominent bands (for example, bands *i*, *ii* and *iii*) corresponding to the larger fragments also contained all of the binding site oligonucleotides. Since the fragments corresponding to bands I and II are short, exist in high molar amount and can be obtained readily in pure form, they were chosen for detailed sequence studies. It may be mentioned here, for example, that although band *iii* exists in Fig. 2a in almost the same radioactive amount as band I, its molar amount is an order of magnitude smaller because it contains a factor of 10 more bases.

For some experiments, fragments were eluted from crushed,

fractionated gels and were studied without further purification. We found that band I and band II materials could, however, be obtained in purer form if the separation of the RNA fragments from rRNA was carried out in two stages.

The RNA obtained from the phenol extraction procedure was first fractionated on a sucrose density gradient without urea treatment. After centrifugation, about half of the radioactivity remained near the top of the gradient while the other half sedimented with the rRNA. Gel electrophoretic analyses showed that fractions from the top of the gradient contained mostly the larger fragments exhibited in Fig. 2a but relatively small amounts of band I and band II material. The 18S RNA fractions contained band I and very little else. The 28S RNA fractions contained band I and band II materials in roughly equal amounts, together with some heterogeneous larger material. Generally, we combined the 18S and 28S fractions, subjected them to gel electrophoresis, and eluted bands I and II for purposes of sequence analysis. Figure 2b shows the gel electrophoresis pattern of RNA fragments obtained in this way. About 0.2% of the radioactivity used for binding was recovered in
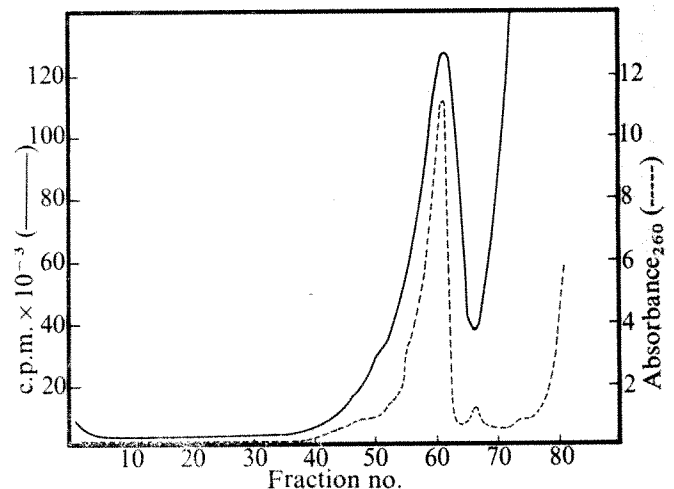
Fig. 1　Binding of the RNA4 fragments to ribosomes. BMV RNA labelled with ^{32}P was prepared as described previously[12,29]. Samples (120 μg; specfiic activity 0.5 μCi per μg RNA) were digested with 0.12 μg of RNase T_1 (enzyme: substrate = 1:1,000) for 15–20 min at 0 °C in 100 μl of buffer consisting of 0.1 M NaCl, 0.01 M magnesium acetate and 0.025 M Tris-HCl (*p*H 7.5). After incubation, the reaction mixture was diluted to 0.5 ml with the above buffer, extracted with an equal volume of phenol at 0–4 °C and precipitated twice with 2 volumes of ethanol. The final precipitate was dissolved in 50 μl of distilled water. Binding of the RNA fragments was carried out in a wheat embryo cell-free extract. A procedure, modified from Davies and Kaesberg[30], was used to prepare a cell-free extract. After homogenisation the 23,000*g* supernatant was made 4 mM in magnesium acetate and preincubated with 20 mM Tris-acetate, 1 mM ATP, 0.06 mM GTP, 10 mM creatine phosphate, 75 μg ml⁻¹ creatine phosphokinase (from Calbiochem, containing 58% protein) and 1 mM dithiothreitol at 30 °C for 20 min. Ten millilitres of the preincubated extract was then passed through a Sephadex G-25 column (2 × 41 cm) equilibrated with 10 mM Tris-acetate, *p*H 7.6, 95 mM potassium acetate, 1 mM magnesium acetate and 1 mM dithiothreitol. Fractions with A_{260} between 40 and 220 were pooled and stored at −90 °C in small aliquots. Binding was performed in a 2 ml reaction mixture containing 100 μg (5 × 10⁷ c.p.m.) of the RNA partial digest, 1 ml S23, 20 mM HEPES, *p*H 7.6, 2.5 mM ATP, 0.38 mM GTP, 10 mM creatine phosphate, 250 μg creatine phosphokinase, 0.04 mM each of the 20 amino acids and 25 μg of anisomycin. The reaction mixture was made 4 mM in magnesium acetate and 95 mM potassium acetate and incubated at 30 °C for 20 min. The mixture was quickly cooled on ice and applied to three 10–40% sucrose density gradients prepared in 50 mM Tris, *p*H 7.6, 50 mM KCl and 4 mM magnesium acetate. The gradients were centrifuged at 4 °C in a Spinco SW27 rotor for 4 h at 27,000 r.p.m. Half millilitre fractions of the gradients were collected manually by puncturing the bottom of the tube and counted. The absorbance at 260 nm was measured in a Cary spectrophotometer. Radioactivity was measured as Cerenkov radiation in a Beckman LS300 scintillation counter.

bands I and II. The band I and band II fragments can be rebound to ribosomes but we have not found conditions to bind them to purified rRNA.

Sequence analysis of the initiator fragments

T_1 and pancreatic RNase fingerprints of the RNA fragments isolated from band I and band II are shown in Fig. 3. The yields and the molar ratios of the oligonucleotides were very consistent from one experiment to another, as would be expected for reproducible binding of a specific BMV RNA4 fragment. The fingerprints of band II RNA contain all the oligonucleotides of those of band I, suggesting that band I contains a BMV RNA4 fragment consisting of a part of that of the band II fragment. The primary sequences of most of the oligonucleotides in the fingerprints were determined by standard methods[14-16]. The sequence of the large oligonucleotide UAUUAAUAAUG at the top of the T_1 fingerprint was determined by two dimensional homochromatography after partial spleen[16] and partial pancreatic RNase digestion followed by base composition analysis of the products. The sequence of the T_1 oligonucleotide XG (corresponding to pancreatic oligonucleotide XGU) requires special comment. Its structure has been determined in collaboration with Dr Fumio Harada. It was found to be m⁷G⁵′ppp⁵′Gp.The evidence for this structure will be described in detail elsewhere but can be summarised as follows: (1) the oligonucleotide XG is completely resistant to digestion with T_2 RNase and pancreatic RNase; (2) treatment with alkaline phosphatase released only 25% of its radioactivity as inorganic phosphate; (3) limited digestion with snake venom phosphodiesterase released 7-methylguanosine-5′-phosphate and ppGp by the scission m⁷G⁵′p/pp⁵′Gp. The products were identified by two dimensional thin layer chromatography[17] and by coelectrophoresis with authentic markers; (4) complete digestion with snake venom phosphodiesterase converted the oligonucleotide into the products: pm⁷G, pGp and P_i, that is, by the scission m⁷G⁵′p/p/p⁵′Gp. This indicates that the m⁷G has a free 3′ hydroxyl and is linked through its 5′-phosphate; (5) nucleotide pyrophosphatase, which can cleave pyrophosphate linkages, cleaved 7-methylguanosine-5′-phosphate from the oligonucleotide, giving the same products as above.

This oligomer is the only T_1 product having a 5′-phosphate and it has been detected as such in the T_1 fingerprint of the BMV RNA4 molecule itself. Periodate oxidation and β-elimination of BMV RNA4 removed the m⁷G, giving rise to a new T_1 product pppGp. All of this evidence indicates that m⁷G has a 3′ hydroxyl group and that the structure m⁷G⁵′ppp⁵′Gp must be present at the 5′-end of the RNA molecule. Thus, the 5′-end of the ribosome-bound fragments comes from the 5′-end of RNA4.

The complete nucleotide sequence of the band II RNA is shown in Fig. 4 as achieved from overlapping sequences in T_1 and pancreatic oligonucleotides, partial T_1 RNase digestion and base analysis of the products. Band I contains the first 22 nucleotides of band II. We have determined the sequence of the first 10 amino acids of *in vitro* synthesised BMV coat protein (Russian strain) by use of an Edman Begg automatic sequencer and in the same manner described for the determination of other parts of the BMV coat protein sequence[18]. The sequence is Ser-Thr-Ser-Gly-Thr-Gly-Lys-Met-Thr-Arg. This sequence is in agreement with that reported for the first 25 amino acids of the wild-type BMV coat protein[19]. The sequence of the first 14 amino acids, together with the nucleotide sequence of band II, is shown in Fig. 4. According to the genetic code, the protein sequence corresponds precisely to the nucleotide sequence following the initiation codon AUG at position 10.

Features of the sequence

In the preceding section we have presented the nucleotide sequence of the portion of BMV RNA4 specifically recognised by wheat embryo ribosomes. Its most distinctive feature is the location of the initiation codon only 10 nucleotides from the 5′- terminus. This is in marked contrast to the situation for the

sequences of bacteriophage RNAs. The initiation codons of the first cistron (A-protein cistron) of phage R17 and phage MS2 RNA are located 130 nucleotides from the 5′-terminus[3,4,20] and those of Qβ RNA are 61 nucleotides from the
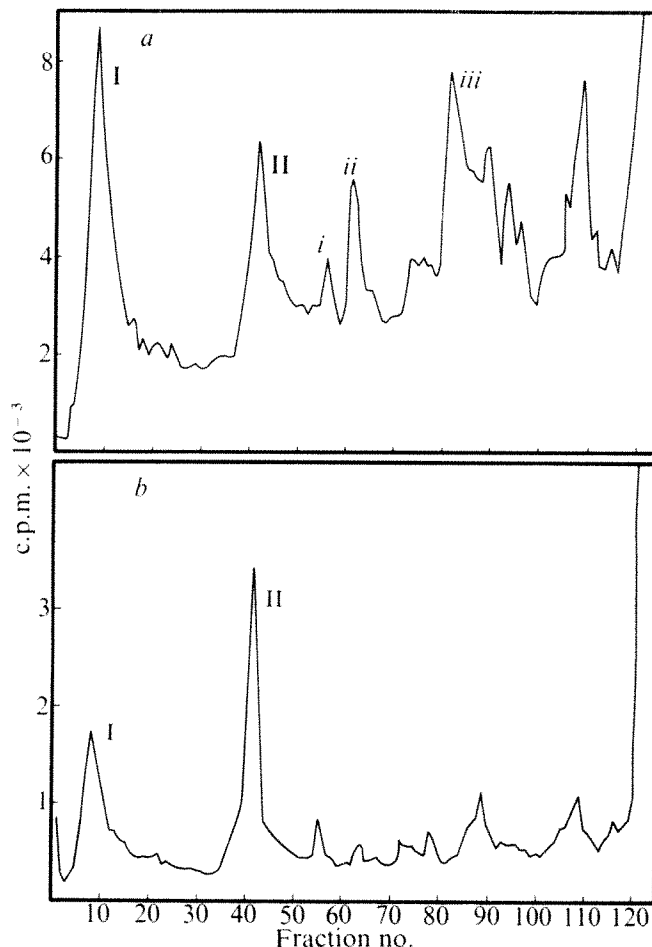
Fig. 2 Gel electrophoresis of RNA fragments isolated from the ribosome binding complex. The fractions from the 80S ribosome region in Fig. 1 were pooled and precipitated with two volumes of ethanol. The pellet was resuspended in 1 ml of 0.2 M Tris-HCl (pH 7.4), 0.2% sodium dodecyl sulphate and extracted with an equal volume of phenol. The RNA was then precipitated with ethanol. *a*, The precipitate was dissolved in 0.5 ml of a suspension buffer containing 0.02 M NaCl, 0.005 M Tris-HCl (pH 8.3) and 6 M urea and the solution was heated at 60 °C for 10 min to dissociate ³²P-labelled RNA from the unlabelled RNA. The solution was cooled to 0 °C and diluted with an equal volume of the suspension buffer without urea. The solution was then applied to a 5–20% sucrose gradient prepared in the suspension buffer containing 3 M urea. The gradient was centrifuged at 4 °C in a Spinco SW27 rotor for 20 h at 26,000 r.p.m. The fractionation of the gradient and the measurement of the absorbance and the radioactivity of each fraction was done as described in Fig. 1. The first 10 fractions from the top were pooled, the RNA was precipitated with ethanol, and dissolved in 50 μl of the TEB buffer of Peacock and Dingman[31] and 5 μl of a solution containing 50% sucrose and 0.1% bromophenol blue was added to it. The sample was then loaded on a 10% polyacrylamide gel and subjected to electrophoresis at a constant current of 3 mA per gel for 2.5 h at room temperature. The gel was crushed in an automatic Gilson gel crusher which crushed gels into fractions corresponding to gel slices 1 mm thick. Electrophoresis was from right to left. *b*, After phenol extraction and ethanol precipitation, the ribosome-bound RNA was dissolved in 0.5 ml of 0.2 M NaCl, 0.05 M Tris-HCl (pH 8.0), and then applied to a 5–20% sucrose gradient made in the same buffer. Centrifugation and fractionation of the gradients were done as described above. The RNA from the 18S and 28S regions was pooled, combined and precipitated with ethanol. The precipitate was dissolved in 0.5 ml of the suspension buffer containing 6 M urea, heated at 60 °C for 10 min and then subjected to sucrose gradient containing 3 M urea as described in (*a*). The ³²P-labelled RNA was isolated and subjected to gel electrophoresis.

5'-terminus[1]. BMV RNA4 has an estimated chain length of approximately 900 bases. Roughly 600 of these bases are assignable to the coat protein cistron[12]. This suggests that one-third of the BMV RNA4 sequence, adjacent to the 3'-end, has an unknown function other than that of encoding a protein sequence.

BMV RNA4 is unusual in another respect. Although it is a monocistronic messenger for the BMV coat protein, this RNA is not required for infectivity. BMV RNAs 1, 2 and 3 are needed for infectivity; RNA4 is regenerated in the infection process and is present in progeny virions. The sequence of RNA4 is a part of the sequence of RNA3 and complementation studies verify that RNA3 contains the coat protein cistron. The coat protein cistron, however, is translated *in vitro* to only a small extent from RNA3, although a second protein, designated protein 3a, is well translated. We infer that *in vivo*, some

Fig. 4 Nucleotide sequence of the ribosome-binding fragment of band II shown with the N-terminal amino acid sequence of the coat protein. T_1 partial products which helped to deduce the sequence are shown by solid lines. These were obtained by a limited digestion of the band I and band II material with an enzyme: substrate ratio of 1:500 for 15 min at 4 °C. The products were fractionated in two dimensions by homochromatography and identified by fingerprint analysis after complete digestion with T_1 RNase. Palindromes are shown by dotted lines.

Fig. 3 RNase fingerprints of ribosome binding fragments of BMV RNA. *a*, T_1 RNase digest of band II; *b*, T_1 RNase digest of band I; *c*, pancreatic RNase digest of band II; *d*, pancreatic RNase digest of band I. Fractions corresponding to band I and band II shown in Fig. 2*b* were pooled and RNA was eluted by shaking the gel particles in 2 ml of 0.25 M NaCl for 3 h at room temperature. Gel particles were removed by Millipore filters and 50 μg of unlabelled yeast RNA was added to the filtrate. RNA was recovered by ethanol precipitation. After one additional ethanol precipitation, each sample was divided into two parts and subjected to fingerprint analysis following standard procedures[14]. For determining the sequence UAUUAAUAAUG, the T_1 oligomer was divided in to two parts and subjected to limited digestion with 2 μl of a solution of spleen phosphodi-esterase (5 mg ml⁻¹; 2 min at room temperature) and pancreatic RNase (50 μg ml⁻¹ containing 5 mg ml⁻¹ unlabelled yeast RNA; 15 min at 4 °C). The products were separated by two-dimensional homochromatography and identified by complete digestion with pancreatic RNase. XG is m⁷G 5' p-p-p 5' Gp. Oligonucleotide AG in the pancreatic fingerprint of band I (*d*) comes from the 3'-end of the fragment

of RNA3 is processed—presumably by nucleolytic cleavage, replication and 5'-terminal modification, to yield RNA4. Thus, a pre-translational cleavage is necessary for expression of the coat protein cistron. It may be mentioned that RNA3 also has a m⁷G 5' p-p-p 5' Gp terminus and it is probably implicated in the effective translation of protein 3a.

The presence of 7-methylguanosine and the pyrophosphate linkage has been recently reported to occur in viral messenger RNA from reovirus[21], vaccinia virus[22,23] and cytoplasmic polyhedrosis virus[24]. It is also present in Novikoff hepatoma cell nuclear RNA[25], and the reovirus genome RNA[26]. Our fingerprint analysis of BMV RNA4 shows that about 50 to 75% of it contains this structure whereas the BMV RNA4 binding fragments contain a quantitative amount. This differ-ence could mean that some of BMV RNA4 lacks part or all of the distinctive 5'-terminus. Possibly ribosomes select those fragments containing it. We find, however, that the oligomer m⁷G 5' p-p-p 5' Gp does not bind significantly to wheat embryo ribosomes so that this structure alone, cannot be responsible for the binding of ribosomes. Our binding results show that the fragment of band I can bind ribosomes as efficiently as the band II fragment. This implies that the ribosome binding signal resides within the first 22 nucleotides. The binding site sequence does not form a stable secondary structure as evaluated accord-ing to Tinoco *et al.*[27]. The association of the initiation fragments with rRNA implies a sequence complementarity between messenger and rRNA[28]. We suggest that the unique 5'-terminus, the short sequence of As and Us, and the AUG codon constitute a simple and efficient ribosome recognition and binding site.

1 Weissmann, C., Billeter, M. A., Goodman, H. M., Hindley, J., and Weber, H., A. Rev. Biochem., 42, 303–328 (1973).
2 Min Jou, W., Haegeman, G., Ysebaert, M., and Fiers, W., Nature, 237, 82–88 (1972).
3 Adams, J. M., Spahr, P. F., and Cory, S., Biochemistry, 11, 976–988 (1972).
4 Steitz, J. A., Nature, 224, 957–964 (1969).

5 Hindley, J., and Staples, D. H., *Nature*, **224**, 964–967 (1969).
6 Gupta, S. L., Chen, J., Schaefer, L., Lengyel, P., and Weissmann, S. M., *Biochem., biophys. Res. Commun.*, **39**, 883–888 (1970).
7 Porter, A. G., Hindley, J., and Billeter, M. A., *Eur. J. Biochem.*, **41**, 413–420 (1974).
8 Pieczenik, G., Model, P., and Robertson, H. D., *J. molec. Biol.*, **90**, 191–214 (1974)).
9 *Methods in Enzymology* (edit. by Moldave, K., and Grossman, L.), **30**, 601–754 (Academic, New York, 1974).
10 Bockstahler, L., and Kaesberg, P., *Nature*, **190**, 192–193 (1961).
11 Lane, L. C., and Kaesberg, P., *Nature new Biol.*, **232**, 40–43 (1971).
12 Shih, D. S., and Kaesberg, P., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1799–1803 (1973).
13 Cundliffe, E., in *Molecular Basis of Antibiotic Action* (edit. by Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., and Waring, M. J.), 278–379 (Wiley Interscience, New York, 1972).
14 Barrell, B. G., in *Procedures in Nucleic Acid Research* (edit. by Cantoni, G. L., and Davies, D. R.), **2**, 751–779 (Harper and Row, New York, 1971).
15 Brownlee, G. G., in *Laboratory Techniques in Biochemistry and Molecular Biology* (edit. by Work, T. S., and Work, E.), **3**, 67–260 (North Holland/American Elsevier, 1972).
16 Siedman, J. G., Comer, M. M., and McClain, W. H., *J. molec. Biol.*, **90**, 677–689 (1974).
17 Saneyoshi, M., Ohashi, Z., Harada, F., and Nishimura, S., *Biochim. biophys. Acta*, **262**, 1–10 (1972).
18 McKean, D. J., Peters, E. H., Waldby, J. I., and Smithies, O., *Biochemistry*, **13**, 3048–3051 (1974).
19 Tremaine, J. H., Ronald, W. P., and Agrawal, H. O., *Proc. Am. Phytopathological Society*, **1**, 83 (1974).
20 DeWatcher, R., Vandenberghe, A., Merregaert, J., Contreras, R., and Fiers, W., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 585–589 (1971).
21 Furuichi, Y., Morgan, M., Muthukrishnan, S., and Shatkin, A. J., *Proc. natn. Acad. Sci. U.S.A*, **72**, 362–366 (1975).
22 Urushibara, T., Furuichi, Y., Nishimura, C., and Miura, K., *FEBS Lett.*, **49**, 385–389 (1975).
23 Wei, C. M., and Moss, B., *Proc. natn. Acad. Sci. U.S.A.*, **72**, 318–322 (1975).
24 Furuichi, Y., and Miura, K., *Nature*, **253**, 374–375 (1975).
25 Reddy, R., Ro-choi, T. S., Henning, D., and Busch, H., *J. biol. Chem.*, **249**, 6486–6494 (1974).
26 Furuichi, Y., Muthukrishnan, S., and Shatkin, A. J., *Proc. natn. Acad. Sci. U.S.A.* **72**, 742–745 (1975).
27 Tinoco, I., Jr, Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M., and Gra-la, J., *Nature new Biol.*, **246**, 40–41 (1973).
28 Shine, J., and Dalgarno, L., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1342–1346 (1974).
29 Bastin, M., and Kaesberg, P., *J. gen. Virol.*, **26**, 321–325 (1975).
30 Davies, J. W., and Kaesberg, P., *J. Virol.*, **12**, 1434–1441 (1973).
31 Peacock, A. C., and Dingman, C. W., *Biochemistry*, **6**, 1818–1827 (1967).

letters to nature

Observations of a transient X-ray source with a period of 104 s

DURING routine observations of the Crab Nebula by Ariel V, experiment A, the rotation modulation collimator (RMC), discovered a new X-ray source A0535+26. The source was just detectable during the first observation on April 13, and during the 16 d of observation was seen to brighten to nearly twice the Crab's intensity (in the 3–7 keV range). The source varies periodically, with a period of 104 s, and has a very flat spectrum in the 3–7 keV range.

Figure 1 shows the observed count rate from A0535+26 as a function of time in units of counts s^{-1} in the 3–7 keV range corrected for the collimator response. Intensities before April 21 were obtained by overlaying several orbits of data together. An approximate ratio between Uhuru counts and the RMC counts is 13:1. The source was definitely present at a low irregular level several days before the main increase started, and it returned to a low level or turned off for short periods during the early stages of increase. This behaviour is reminiscent of that shown by A1118−61 (refs 1, 2) which returned to a low level twice in the period immediately before the main

increase in intensity. But the smaller deviations from a smooth curve during the latter part of the observations are probably a result of interference from the Crab X-ray source which was close to the spin axis at the time and are probably not significant. Thus the later behaviour differs from the continued irregular behaviour of A1118−61. Data from another experiment[3] on Ariel V indicate that the source reached a peak within a few days after our observations ceased. Though the rise time is slow compared with optical novae and supernovae, it is comparable with other X-ray transients.

The position of A0535+26 has been measured to be 05h35min47s±2s +26°16'52"±45" (1950.0) (90% confidence) which differs from our previously announced position[4] by 2'. The RMC determines positions relative to the spin axis pointing position of the spacecraft. Using the relative position of the Crab Nebula from our data, and its position on the sky taken to be 15" NW of the radio pulsar[5], the attitude of the spin axis was determined independently of the crude spacecraft attitude data. One additional parameter is necessary from the spacecraft, the azimuth angle of the arbitrary starting point of its rotation. At the beginning of the Crab Nebula observations new spacecraft alignment parameters were used to correct for

Fig. 1 Light curve of A0535+26. Bars indicate periods of integration (where these exceed one orbit) and ±1σ errors. Upper limits are 3σ.
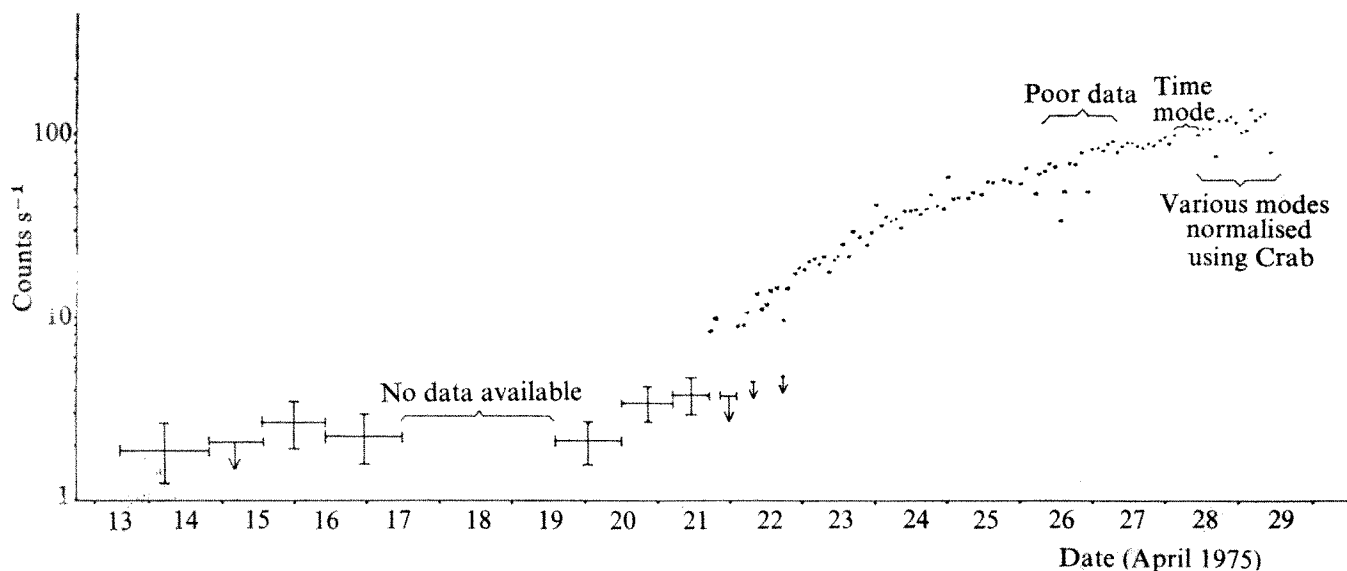
previously large (~0.5°) systematic errors in the spacecraft attitude solutions. But it was not until after the Crab Nebula observations (in the Cygnus region using Cyg X-1 and Cyg X-3 as benchmarks) that it was found that the new alignment parameters caused a change in azimuth angle (zero sector angle) of 20'. This in turn caused a change in right ascension at the position of A0535+26 of 2'.

During four orbits on April 28, 1975, data were collected in the experiment's "time" mode. In this mode data are integrated for 32 s and stored. Each orbit yielded about 45 min of useful data, which were collected starting at 4.21695, 5.80538, 7.390116 and 8.99238 h UT. The average count in 32 s was 6,000, made up by a 40% contribution due to A0535+26, and 30% each due to the Crab and to total particle and diffuse X-ray background in the 17° FWHM field of view. The sum of the power spectra for all four orbits of time mode data is shown in Fig. 2. This power spectrum differs from that expected from a constant signal with statistical noise fluctuations in three ways: (1) an excess low frequency component; (2) excess power at all frequencies as compared to expected statistical fluctuations; and (3) marked sinusoidal features at 0.960×10^{-2} Hz (104.1 s) and 1.20×10^{-2} Hz (83.3 s).

The low frequency component is a result of a combination of slow drift in the intensity of the source and background, and
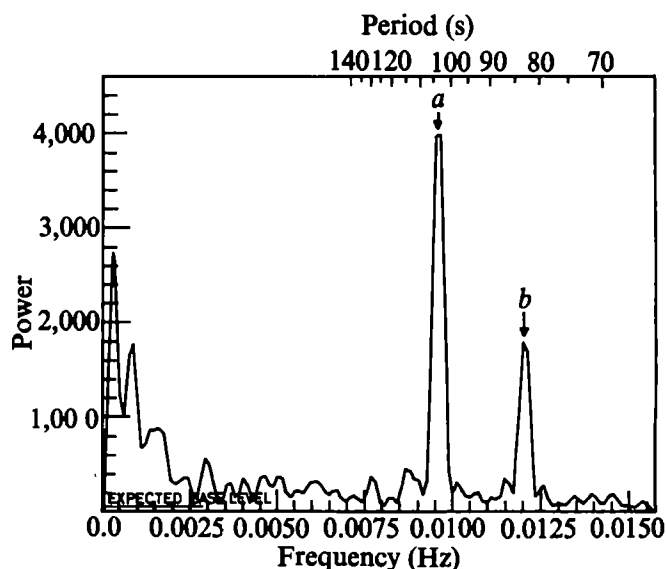
Fig. 2 Sum of power spectra of the four orbits in TIME mode. Only the mean has been removed. The height of the 104.1-s peak corresponds to a 25% modulation of the source, and its width is that expected for a pure sine wave. *a*, 104.1 s; *b*, 83.3 s.

Table 1 Possible periods which would be aliased to 104.14 s with a sampling time of 32 s

Period (s)	Frequency (Hz)	Attenuation factor
104.14	0.96×10^{-2}	0.852
46.1	2.17	0.376
24.4	4.09	−0.201
18.9	5.29	−0.154
13.9	7.21	0.112
11.9	8.42	0.098
9.7	10.34	−0.078
8 7	11.54	−0.073
7.4	13.46	0 063
6.8	14,66	0.054

The attenuation factor caused by the finite sampling is also shown.

to the finite sampling time (45 min). Removing a quadratic trend in the data, from each orbit independently, reduces the low frequency component. The excess power over all frequencies indicates that the source is randomly fluctuating over all periods between several seconds to minutes at least.

The periodic feature at 0.960×10^{-2} Hz must be accepted as real, and not an artefact of some type of beating phenomenon between the modulation caused by the rotating grids and the sampling frequency. Since the rotation rate of the spacecraft is nominally 10 r.p.m. the 32-s integration time represents an integration over more than 10 half-rotations of the RMC. Analysis of time mode data from the region of the galactic centre and Cyg X-1 taken before and after the observations reported here do not show any periodic components, nor any comparable power over the measured range of frequencies.

But it is not necessary that the 104.1 and 83.3 s periods are the true incident periods. Because of the integration time of 32 s any frequency above the Nyquist frequency 1/64 s⁻¹ would be aliased to a measured period greater than 64 s. Indeed the 83.3 s period is explained as the 52 s harmonic of the 104 s fundamental aliased to 83.3 s. Furthermore, the 104 and 52 s periodicities could also be aliases of shorter period oscillations. Table 1 lists the first 10 possible higher periods which could be aliased to 104 s with a sampling rate of 32 s. The second harmonic of any of these frequencies would be aliased to produce an apparent period of 83.3 s.

But there is a means of differentiating between some of the possible different frequencies. Since data are integrated for 32 s

the observed periodic signal will have been attenuated by a factor depending on its frequency. The attenuation factors for the possible fundamental frequencies are shown in Table 1. The observed modulation of the signal was 25% of the mean. The shorter periods (24.4 s and less) are precluded because they would imply a modulation depth of the incident signal greater than 100%.

An epoch-folding analysis yields the best estimate for the period of 104.14 ± 0.16 s (1σ). Figure 3 shows the observed light curve for the 104.14 s periodicity, folded into 10 bins for all 340 32-s samples over the four orbits. The third harmonic, if it exists, is aliased near the DC level and is masked by any slow trends in the data. The fourth harmonic is too weak to be seen above the noise. Because of the relatively long sampling time, and thus the low sensitivity to the higher harmonics, no attempt has been made to deconvolve the measured light curve with the instrumental response.

The light curve of the source (Fig. 1) shows no sign of eclipses; the time mode data are insufficient to measure a change in period of the dwarf in its orbit.

Fig. 3 The light curve of the 104.14-s period folded into 10 bins, shown over two cycles. The error shown is statistical. ———, 32-s sample time.
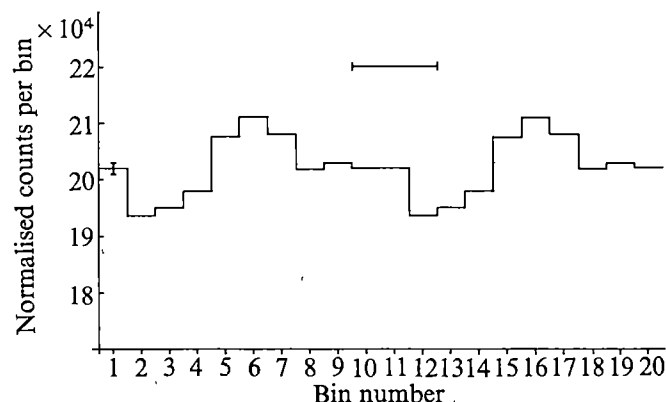
Crude three channel energy resolution (3–7.5 keV) shows that around its peak the spectrum of this source is very similar to the transient A1118–61 (ref. 2). There are indications that during the growth phase the absorption increased to this value from a low level.

F. D. ROSENBERG

Mullard Space Science Laboratory,
University College, London, UK

C. J. EYLES
G. K. SKINNER
A. P. WILLMORE

Department of Space Research
University of Birmingham, UK

1 Eyles, C. J., Skinner, G. K., Willmore, A. P., and Rosenberg, F. D., *Nature*, 254, 577, (1975).
2 Ives, J. C., Sanford, P. W., and Bell-Burnell, S. J., *Nature*, 254, 578 (1975).
3 Ricketts, M. J., Turner, M. J. L., Page, C. E., and Pounds, K. A., *Nature*, (1975).
4 Eyles, C. J., Skinner, G. K., Willmore, A. P., and Rosenberg, F. D., *IAU Circ.*, No. 2274 (1975).
5 Staubert, R., et al., *Astrophys. J. Lett.* (in the press).

Hard X-ray measurements of nova A0535+26 in Taurus

MEASUREMENTS of high energy X rays in the region of the Crab Nebula were made with the Imperial College detector on Ariel V between April 13 and April 29, 1975. During that time the X-ray nova A0535+26 was first detected and observed to rise to its maximum intensity[1,2]. At 30 keV this nova reached an intensity considerably brighter than the Crab Nebula, making it the brightest known source in the sky.

Our experiment uses an actively shielded crystal scintillator, and operated in the energy range 26 keV–1.2 MeV, making
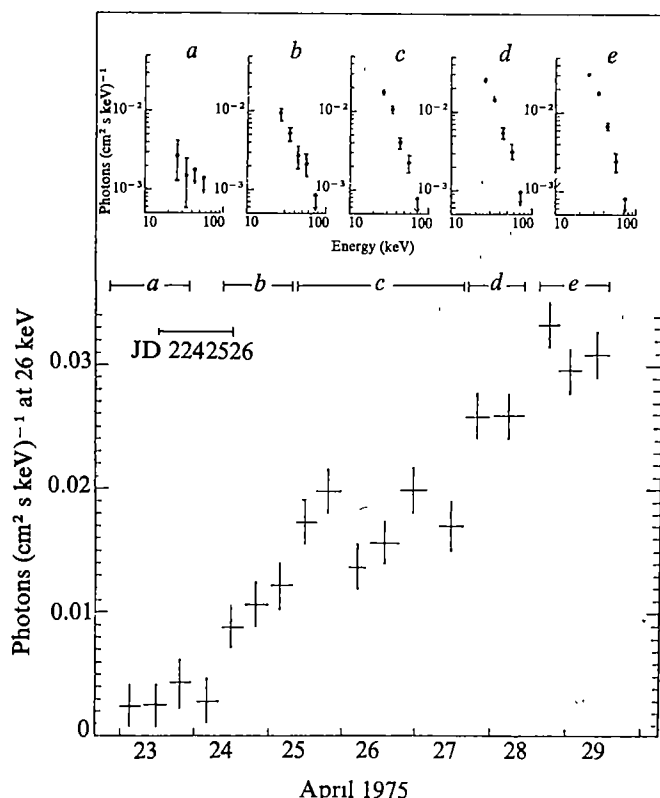
Fig. 1 Light curve of nova A0535+26 obtained from channel 1 of the high energy experiment, with an 8-h time resolution. Data have been normalised to 26 keV. Errors are ±1σ counting statistics. a–e, Five spectra obtained during this period; the data collection period for each spectrum is indicated.

spectral measurements in 16 logarithmically spaced energy channels. It has a sensitive area of 8 cm² and an opening angle of 8° FWHM which is offset from the spacecraft spin axis by 3°. A source a few degrees from the spin axis direction can be detected by determining the modulation in the counting rate accumulated in four equal angle spin sectors. Since a spurious modulation is induced in the apparatus by cosmic rays, a separate measurement is required to subtract this background. This is normally achieved by making observations at offsets on either side of the source; however, in the case of the nova discussed here, data for background subtraction were taken from a period before the nova had reached a significant intensity as measured by the low energy experiments.

For most of the observations reported here, the spin axis was pointing to within 0.5° of the Crab Nebula which does not give any significant modulation for this offset angle. Data for
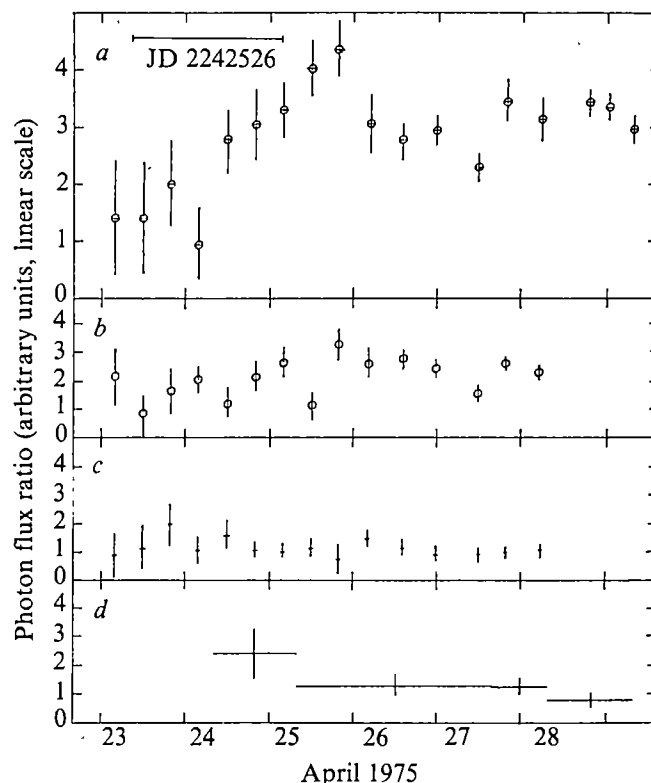
Fig. 2 Photon flux ratios at various energies plotted to indicate any spectral changes during the rise of the event. a–c, Ratio of the fluxes observed in channels 1–3, respectively, to those measured by the RMC experiment, d, the ratio of the flux in channel 4 to that in channel 1 of the high energy experiment. Error bars indicate ±1σ in counting statistics. Energy intervals: RMC experiment, 2.95–7.60 keV; our experiment: channel 1, 26–33 keV; channel 2, 33–43 keV; channel 3, 43–56 keV; channel 4, 56–73 keV.

background subtraction were obtained on April 20, 1975, and it is assumed that the nova source had not reached a detectable level for this experiment on that day. The position of the nova[1] places it 4° from the spin axis which happens to be the offset giving maximum modulation and therefore maximum sensitivity for this experiment. For the last day of the observation the spin axis was offset 1.4° from the Crab Nebula towards the nova and corrections had to be applied, first for the signal now produced by the Crab Nebula and second for the slight reduction in efficiency because of the reduced offset from the nova. Data from previous measurements on the Crab Nebula were used to make these corrections.

Figure 1 shows the light curve obtained from the lowest energy channel with an 8-h time resolution starting when the first statistically significant signal was observed. The flux has

been corrected to the lower threshold of this channel (26 keV). The phase of the spin modulation for this data corresponds to the stated position of the nova; any spurious signal originating from the Crab Nebula would change this phase, which is kept as a free parameter in the analysis.

The light curve has been divided into five sections and data from each section used to obtain energy spectra. (Fig. 1a–e). Upper limits are shown at a 2σ significance level. The spectra show some evidence of steepening as the nova brightens.

Further information on the spectral development is given in Fig. 2 where results from this experiment are plotted as a ratio to the low energy measurements made with the rotation modulation collimator (RMC) experiment described in ref. 1.

Whereas the channel 1: RMC ratio (Fig. 2a) indicates some initial hardening of the spectrum the channel 4 : channel 1 ratio (Fig. 2d) shows a gradual softening. The latter may also be noted in Fig. 1a–e where, after the initial section, the flux at 56 keV remains essentially constant whereas at 26 keV it increases by a factor of 3. But though the low energy intensity increases by a factor of 10 during the period covered, the results do not indicate any dramatic change in the spectrum.

In Fig. 3 we have combined the spectrum from the high energy experiment taken in period d (see Fig. 1) with three low energy points from the RMC experiment taken in the same period. A simple power law will not fit the data in the range shown. The results can be interpreted in terms of accretion of material from a normal star on to a compact object like a neutron star. Suppose that the X-ray flare-up is caused by a large change in the amount of accretion material available.

Fig. 3 Spectrum obtained by combining data from the high energy experiment (lower five points) with those from the RMC experiment (upper three points) measured during the same period (Fig. 1d). Errors are $\pm\, 1\sigma$ and 2σ for the upper limit based on counting statistics. a, 3 keV black body curve; b, 8 keV modified black body curve.
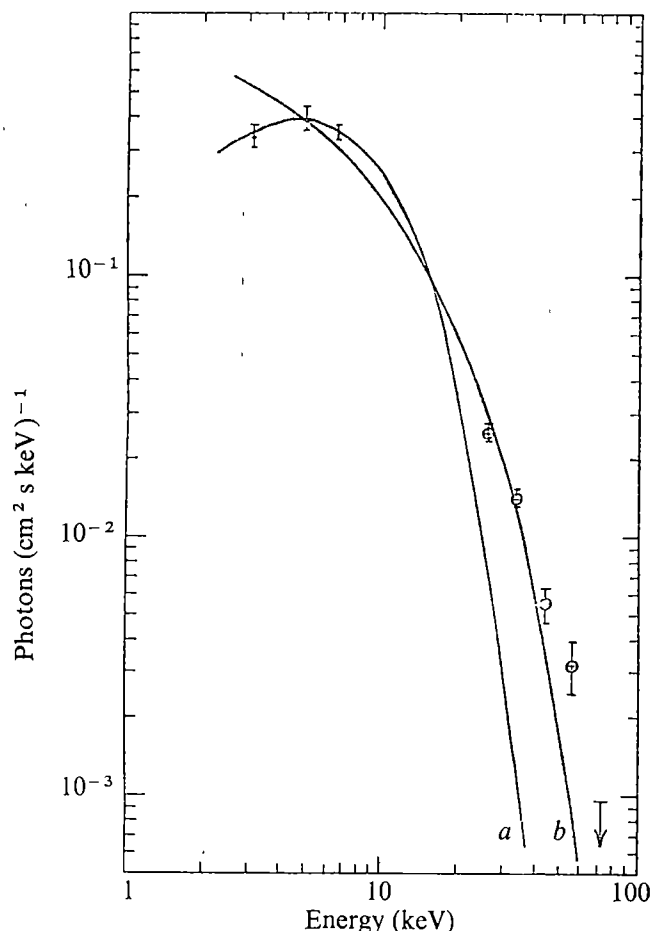
The outer layers of the accretion disk may be expected to emit low energy X rays according to a black body photon spectrum:

$$(dN/dE)_{BB} \propto E^2/[\exp(E/kT)-1]$$

More energetic photons produced in the deeper layers will emerge after scattering by electrons in a finite skin depth. The photon spectrum expected for this situation is [3,4]:

$$(dN/dE)_M \propto (dN/dE)_{BB}\{g[1-\exp(-E/kT)]/E^3\}^{\frac{1}{2}}$$

with a Gaunt factor $g \propto E^{-0.4}$.

The deepest layers, which correspond to the greatest gravitational energy, may radiate according to a thermal bremsstrahlung law, but the overlaying layers will only be transparent to the high energy tail.

Whereas a black body curve with $kT = 3\pm0.5$ keV fits the low energy data, a modified black body curve with $kT = 8\pm1$ keV is required for the high energy data. No simple single parameter curve will fit all the data. Our results therefore point to a multilayer, multitemperature source model.

Suppose that the increase in source intensity is attributable to an increasing flux, F, of material accreting on to a compact object. Then, for black body radiation, a consequence of Stefan's Law and the transfer of gravitational energy to thermal energy in a constant accretion disk geometry implies that

$$T_{BB} \propto F^{\frac{1}{4}}$$

and thus the spectral shape will only be weakly dependent on T_{BB}. Pringle and Rees have also found[4] a weak dependence on F of both the modified black body temperature and the point of transition between the two laws. The relatively constant shape of the spectrum during the flare-up of the source is thus consistent with an accretion model in a binary system.

We thank the groups at the Mullard Space Science Laboratory and the University of Birmingham for making results from the RMC experiment available to us.

M. J. Coe
G. F. Carpenter
A. R. Engel
J. J. Quenby

Physics Department,
Imperial College,
London SW7 2AZ, UK

[1] Rosenberg, F. D , Eyles, C. J., Skinner, G. K., and Willmore, A. P., *Nature*, **256**, 628–630 (1975).
[2] Ricketts, M. J., Turner, M J. L., Page, C G., and Pounds, K. A., *Nature*, **256**, 631–633 (1975).
[3] Felton, J E , and Rees, M J., *Astr Astrophys* , **17**, 226 (1972).
[4] Pringle, J. E., and Rees, M. J., *Astr. Astrophys* , **21**, 1 (1972).

Observations of A0535+26 with the Leicester sky survey experiment

THE Leicester University Sky Survey instrument with its field of view perpendicular to the spin axis was able to continue the study of A0535+26 after the spin axis experiments (see accompanying papers) were manoeuvred away on April 29. The source was close to the centre of our field of view on May 2, May 19 and June 1. On the first occasion the instrument was in a single channel mode (no spectral information) but eight-channel spectra between 2 and 18 keV were obtained on the second and third observations.

In addition to these three direct observations a measurable signal was obtained continuously from May 3 to May 31 while the source was outside the normal field of view of the instrument (FWHM 10°). This resulted from the brightness of the source at the higher energies; the detector efficiency combined
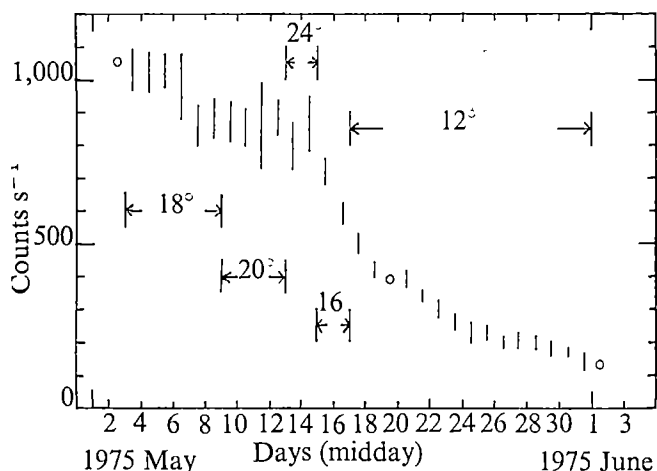
Fig. 1 The light curve of A0535+26 obtained with the Leicester University Sky Survey instrument. Points are mean daily intensities, errors are one sigma poissonian. The offsets at which the data were collected are indicated in degrees.

Table 1 Spectral parameters for $I = CE^{-\alpha}$ photons $(cm^2\ s\ keV)^{-1}$

Date	Nova Coefficient C	Index α	Crab Nebula Coefficient C	Index α
May 19	1.62	−0.8	12.0	−2.1
June 1	0.67	−1.1	10.2	−1.95

Statistical error in index is 0.1.

across a manoeuvre. The resulting light curve, consisting of daily averages, is shown in Fig. 1. On May 2, we find the intensity (3–18 keV) to be three times that of the Crab Nebula. Direct comparison with the pre-maximum light curve of the MSSL/Birmingham Modulation Collimator experiment (accompanying paper) must take account of the spectral hardness of the A0535+26 source since in the energy band of that experiment (2–8 keV) the relative strength of the transient source is less pronounced.

The peak intensity of A0535+26 seems to lie between the periods covered by the two light curves, occurring between April 29 and May 2. The main features are an initial slow fall, to May 16, followed by a sharper decrease in intensity, which is apparently close to exponential, over the following 15 d.

with the transmission through the wall of the collimator gives a mean response of ~1% at 12° offset decreasing to ~0.3% at 28° offset, for the observed spectrum of A0535+26. In this way, it has been possible to extend the Ariel V measurements of intensity to cover the whole period from the appearance of the source to June 1, leaving a gap of two days from April 29 to May 2.

The data from the various large offset observations were combined to give a continuous light curve. The method used was to correct those large angle measurements with adjacent direct measurements so that they fitted smoothly. For the data obtained between May 9 and May 17 no direct comparison was available and these data have therefore been adjusted so that the intensity changes smoothly from one offset to the next

Fig. 2 The two sets of eight-channel spectral data from A0535+26, together with predicted counts for the best fit power law spectrum. +, Observation with 1σ error; − − −, best fit prediction.

Fig. 3 The best fit input spectra with the deduced input photon spectrum. The May 19 spectrum is shown with (dashed) the effect of a low energy cutoff at 1.2 keV. *a*, May 19 with $E^{-0.8}$; *b*, June 1 with $E^{-1.1}$.

Thirty seconds of exposure to A0535+26 were obtained on May 19 in PHA mode, and 40 s on June 1. Both observations were timed to coincide with balloon observations of the source with high energy X-ray instruments (of Southampton University and MIT).

Spectral data for the two observations are plotted in Fig. 2, and Table 1 lists the optimum spectral parameters, together with those for the Crab Nebula measured simultaneously. The best fitting input spectra of A0535+26 are also shown in Fig. 3, here the relative measured intensities are given for each energy

channel, determined by comparing the observations with the input spectra folded through the detector response.

The spectrum obtained on May 19 is best characterised by a power law index of -0.8 with no absorption. The upper limit for the cutoff energy (1.2 keV) corresponds to a hydrogen column density of less than 10^{22} atom cm^{-2}. It must be emphasised that the power law fit over such a restricted energy range does not imply a non-thermal source mechanism and is used only to allow a convenient description of the spectral parameters. The June 1 spectrum is best fitted with a somewhat steeper power law of index 1.1, again with no measurable low energy attenuation. Thus if the spectrum is thermal it seems that the emission region has cooled significantly over a thirteen day period. Spectral data from the MSSL proportional counter viewing along the satellite spin axis is reported (J. Ives and J. Burnell, private communication) to show strong low energy attenuation during the rising part of the light curve, indicating that in this respect also the spectrum has changed significantly during the evolution of A0535+26.

M. J. Ricketts
M. J. L. Turner ·
C. G. Page
K. A. Pounds

X-Ray Astronomy Group,
Department of Physics,
University of Leicester, UK

[1] Brown, R. L , and Gould, J., *Phys. Rev. D.*, 1, 2252 (1970).

Decay of X-ray source A0535+26

THE All-Sky Monitor (ASM) aboard Ariel V is a pinhole camera sensitive to X rays in the energy range 3–6 keV. It has an effective area of 0.6 cm² with a duty cycle of $\sim 1\%$ for a given source, and monitors more than 3π of the celestial sphere each orbit[1]. It is important to note that the regions of sky inaccessible to the ASM include the spacecraft spin axis and a band about the spacecraft equator, so that its field of view and those of the other five Ariel V experiments are mutually exclusive.

The transient source A0535+26 was first detected by another Ariel V experiment[2] during an extended spin axis hold in Taurus between days 103 and 119 of 1975, during which time a threefold increase in the emission from Cyg X-1 was observed[3,4]. When the spin axis was moved toward Cyg X-1, the Taurus region became accessible to the ASM, enabling us to study the decay of the transient source. When Cyg X-1 was next observed by the ASM from day 136, it had returned to its usual intensity of $\sim 1/3$ the Crab Nebula in the energy band 3–6 keV.

Figure 1 is the overall 3–6 keV light curve of A0535+26 when it was in the field of view of the ASM. Before spin axis hold in Taurus, the new source was not observable at a 2σ upper limit of $\sim 10\%$ of the Crab Nebula intensity (the latter was simultaneously measured at its stationary value of 1.4 cm⁻² s⁻¹). When first observable by the ASM on day 119, (April 29) the new source was $\sim 15\%$ more intense than the Crab Nebula, and stayed at this level for approximately one week. The source then decayed with an e-folding time of ~ 19 d until the Sun was too close to the source for unambiguous separation. When the Sun moved sufficiently far to allow the intensity of the source to again be interrogated, it was found to be unobservable (at $<10\%$ of the Crab Nebula).

The plateau region of the decay measured before day 126 is not unusual for transient X-ray sources. Similar plateau

effects in the decay just after maximum have been observed in the older transient sources Cen X-4 (ref. 5) and 2U1543–47 (ref. 6), as well as in the Ariel V transient source A1524–62 (ref. 7). Unlike these sources, however, the e-folding time for the Taurus source is considerably shorter than the $\gtrsim 2$ months which is typical of the others. The only other transient sources previously reported with comparably short lifetimes are 3U1735 −28 and A1118−61, with which the Taurus source shares spectral and temporal (pulsing) similarities (see accompanying papers and ref. 8). No temporal resolution finer than 100 min is possible with the ASM, nor is there any spectral resolution obtainable in the 3–6 keV acceptance window. The window is, however, insensitive to input spectral form[1], so that the light curve displayed for this band in Fig. 1 does not suffer from any spectrum-dependent systematic offset.
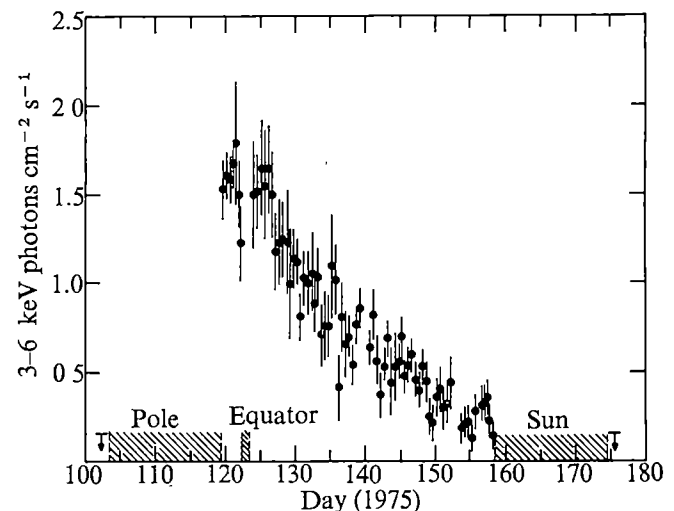
Fig. 1 Intensity measured from A0535+26 over approximately half-day accumulation times. The source is not in the usable field of view of the ASM where the abscissa is shaded. The 2σ upper limits before and after the measured points are determined primarily by the statistical accuracy in the measurement of the Crab Nebula, with which A0535+26 may be confused in the ASM data.

Finally, we note that the short-lived transients typified by 3U1735−28, A1118−61 and A0535+26 may be as much as an order of magnitude more frequent than longer-lived transients. The proximity to the galactic plane argues that these sources have a Population I distribution, and are >1 kpc distant. As discussed in ref. 7, their short lifetimes (and appearances in relatively congested regions of the plane) make them less easily detectable than the longer-lived transients. With an average luminosity at maximum of $\sim 10^{37}$ erg s⁻¹, their frequency may approach 100 yr⁻¹ in the Galaxy without conflicting with existing source or galactic ridge measurements. L. J. K. acknowledges support from the University of Maryland.

L. J. Kaluzienski
S. S. Holt
E. A. Boldt
P. J. Serlemitsos

Laboratory for High Energy Astrophysics,
NASA/Goddard Space Flight Center,
Greenbelt, Maryland 20771

[1] Holt, S. S., *Proc. Cospar Symp. Fast Transients X and Gamma Ray Astronomy, Astrophys. Space Sci* , (in the press)
[2] Eyles, C. J., *et al IAU Circ. No. 2774* (1975).
[3] Gursky, H., *et al., IAU Circ. No. 2778* (1975).

[4] Holt, S. S., Boldt, E. A., Kaluzienski, L. J., and Serlemitsos, P. J., *Nature*, **256**, 108 (1975).

[5] Evans, W. D., Belian, R. D., and Conner, J. P., *Astrophys. J Lett.*, **159**, L57 (1970).

[6] Matilsky, T. A., Giacconi, R., Gursky, H, Kellogg, E. M, and Tananbaum, H. D., *Astrophys. J Lett*, **174**, L53 (1972).

[7] Kaluzienski, L. J., et al., *Astrophys. J.* (in the press).

[8] Ives, J. C., Sanford, P. W., and Bell-Burnell, S. J., *Nature*, **254**, 578 (1975).

Dynamo generation in Mercury

OBSERVATIONS during the most recent encounter of the planet Mercury by Mariner 10 seem to have confirmed the existence of a small but intrinsic magnetic dipole of about 3×10^{-3} gauss $R_M{}^3$, where R_M is the radius of Mercury[1]. This field may be small enough to have a non-dynamo explanation[1,2], but it is nevertheless of interest to examine whether dynamo generation is possible for the present Mercury.

Siegfried and Solomon[3] have calculated models for the interior and thermal evolution of Mercury. In these models, the cosmochemical calculations of Lewis[4] were used to constrain the composition. They concluded that Mercury is most likely differentiated[5], with a Fe–Ni core extending out to about $0.7R_M$, and a silicate mantle. I have tested their models for four necessary (but possibly not sufficient) conditions for magnetohydrodynamic dynamo generation.

First, dynamo generation requires that the interior be at least partially fluid, since solid-state convection is too slow for dynamo generation. In the models of Siegfried and Solomon, radioactive heat sources in the silicate mantle may be sufficient for fluidity in a region extending from about 200 km to about 800 km below the planet surface. The metallic core is either partly or entirely solid, depending on when differentiation took place.

Second, dynamo generation requires an energy source that drives a flow of the fluid relative to a rigidly rotating planet. Thermal convection seems to be the only plausible energy source, since precession is insignificant[2], and the other possible sources are just as unsatisfactory for Mercury as they are for the Earth[6]. The Siegfried and Solomon models assume that all the radioactive heat sources are in the mantle, so that thermal convection occurs in a 600 km thick silicate layer, but not in the stably stratified metallic core. Other heat sources in the core (latent heat, gravitation) seem to be insufficient to drive the temperature gradient superadiabatic and initiate convection. The thermal conductivity of the metallic core is sufficiently high that convection would only occur if about half of the radioactive heat sources reside in the core. One possiblity is that Mercury has a similar fraction of ^{40}K as Lewis[4] proposes for the Earth's core. This would violate Lewis's cosmochemical arguments, since potassium compounds are too volatile to condense from the primitive solar nebula at Mercury's distance from the Sun.

Third, dynamo generation requires that the magnetic field diffusion time, τ_M, exceeds the characteristic fluid flow (convective) time scale τ_C. The ratio of τ_M to τ_C is known as the magnetic Reynold's number. In the convecting silicate layer, simple mixing length theory[7] predicts a convective velocity of 0.1 cm s^{-1} for the expected heat flux[3] of about 50 erg cm^{-2} s^{-1}. A similar estimate applies to the Earth's outer core[8] and is unlikely to be incorrect by more than an order of magnitude. The corresponding convective time scale is $\tau_C \sim$ 1–10 yr. The magnetic field diffusion time is given by[2]

$$\tau_M \approx 10^{-9} \sigma l^2 \text{ s} \tag{1}$$

where σ is the electrical conductivity in Ω^{-1} cm^{-1} and l is the smallest dimension of the convection region. The requirement $\tau_M > \tau_C$ implies $\sigma > 10$–100 Ω^{-1} cm^{-1}. This would be easily satisfied in the core ($\sigma \sim 10^3$ or 10^4 Ω^{-1} cm^{-1}) but not in the semiconducting silicates. In the Siegfried and Solomon models,

the temperature is at most about 2,100 K in the mantle. At this temperature, the conductivity in the Earth is probably less than 0.1 Ω^{-1} cm^{-1} (ref. 9). Cosmochemical arguments[4] indicate that the Mercurian mantle should be somewhat different from the Earth, with MgSiO$_3$ prominent, but a generous extrapolation of high temperature laboratory experiments on enstatite[10], indicates that a conductivity in excess of 1 Ω^{-1} cm^{-1} is very unlikely. This figure is for solid materials, but these minerals (unlike pure germanium or silicon) are not likely to change their short range order or conductivity dramatically upon melting[11]. (The conductivity may increase by as much as an order of magnitude, but certainly not several orders of magnitude.) I conclude that the liquid silicate conductivity is probably inadequate for dynamo generation. I note that generation in a thin silicate layer near the surface of the planet is likely to lead to a substantially non-dipolar field at the planetary surface.

Fourth, dynamo generation requires a fluid flow of sufficient complexity to satisfy Cowling's theorem[12]. In particular, the requirement of a non-axisymmetric field is usually interpreted to imply a need for "rapid rotation" of the planet (although other alternatives may exist[13]). Actually, "rapid rotation" only means that the Coriolis force has an important effect on convective flow. A measure of this is the ratio of inertial to Coriolis forces, $v/\Omega l$, where $v \sim 0.1$ cm s^{-1} is a typical convective velocity in the absence of rotation, and $\Omega \sim 1 \times 10^{-6}$ s^{-1} is the planetary angular velocity. For $l \lesssim 10^5$ cm, this ratio (which is a nominal Rossby number for the flow) is less than unity, and the Coriolis force is important. For the largest scale convective flows in Mercury, $l \approx 10^7$ cm, so that in this respect Mercury is a rapidly rotating planet. The requirements of Cowling's theorem may then be satisfied because of the effect of planetary rotation alone.

In a more complete theory, such as that attempted by Parker[14], and more recently in rigorous work by Busse (unpublished), the criterion for dynamo generation is generally more stringent than the simple ones we have discussed. A complete theory would also make a prediction for the field magnitude. A feature of the Busse theory is that if the core is convecting, then the small field of Mercury would be a consequence of the slow planetary rotation. Unlike the Earth, the ohmic dissipation in Mercury is likely to be completely insignificant compared with any important energy source such as radioactivity.

I conclude that a literal interpretation of the cosmochemical calculations of Lewis[4] implies that dynamo generation in Mercury is improbable. Generation would be possible if the metallic core were contaminated with substantial amounts of radioactive material. It would then follow that not all the material that comprises Mercury condensed from the solar nebula at the present distance of Mercury from the Sun. The magnitude of the field and any future multipolar analysis (such as might be achieved by an orbiter) will be important in determining the field source.

D. J. STEVENSON

Laboratory of Atomic and Solid State Physics,
Cornell University, Ithaca, New York 14853

[1] Ness, N. F., Behannon, K. W., Lepping, R P, and Whang, Y. C., *Nature*, **255**, 204–205 (1975).

[2] Stevenson, D. J, *Icarus*, **22**, 403–415 (1974).

[3] Siegfried II, R. W., and Solomon, S. C, *Icarus*, **23**, 192–205 (1974).

[4] Lewis, J. S., *Earth planet Sci. Lett.*, **15**, 286–290 (1972).

[5] Murray, B. C., et al., *Science*, **185**, 169–178 (1974).

[6] Busse, F. H., *J. geophys. Res*, **80**, 278–280 (1975).

[7] Schwarzchild, M., *Structure and Evolution of Stars*, 47 (Princeton University Press, Princeton, 1958).

[8] Frazer, N., *Geophys J. R. astr. Soc.*, **34**, 193–201 (1973)

[9] Stacey, F. D, *Physics of the Earth*, 144 (Wiley, New York, 1969).

[10] Dvorak, Z. and Schloessin, H. H., *Geophysics*, **38**, 25–36 (1973)

[11] Gubanov, A. I, *Quantum Electron Theory of Amorphous Conductors*, (Consultants Bureau, New York, 1965)

[12] Cowling, T. A., *Mon. Not. R. astr. Soc.*, **94**, 39–48 (1934)

[13] Busse, F. H., *J. fluid Mech.*, **57**, 529–544 (1973).

[14] Parker, E. N., *Astrophys. J.*, **164**, 491–509 (1971).

Fracture mechanism of Henbury meteorite by separation along surfaces of shear faulting

THE smallest Henbury meteorite crater contained[1] a massive iron that had broken into four pieces weighing 132.7, 54.4, 10.9 and 2.3 kg, respectively. When these four pieces were excavated from the depths of the crater they were found to be still juxtaposed to one another. We have studied a 5 kg, 20×40 cm slice (BM 1932, 1359) taken from the largest of these pieces. We have also prepared and studied sections cut from a number of pieces of 'shrapnel' from a Henbury crater, weighing about 100 g and purchased from the American Meteorite Laboratory.

The macrosection BM 1932, 1359 is traversed by a number of fault displacements, most of which are continuous across the whole section and lie approximately parallel to an outer edge of the section. This suggests that, in this instance, the fracture surfaces could represent the extreme situation in which limited shear displacement gives way to physical separation along the fault surface. This macrosection is too large for satisfactory microscopic examination, and, consequently, the fracture mechanism was explored using the smaller shrapnel fragments. In each instance slip-fault displacements were encountered within the shrapnel pieces, and in one instance an internal crack was found to merge continuously into a pronounced displacement fault. The most convincing evidence for fracture by separation along the surfaces of shear faults, however, comes from those pieces of shrapnel that were not reheated significantly during break-up. In those cases, the bulk of the kamacite (αFe–Ni) is shock hardened but not recrystallised. Shear-displacement surfaces within these specimens were revealed (by Nital etch) as continuous traces of fine grained (1–5 μm) recrystallisation in the kamacite, similar to geological mylonitisation zones. The 'throw' of the fault varies from a few micrometres to several millimetres and is best seen where particles of taenite (γFe–Ni) are cut and displaced. The Nital etch did not reveal recrystallisation in the taenite and, consequently, the metallographically visible 'thickness' of the shear effect in taenite was limited to the apparently sharp geometrical surface of displacement. But the heat that is generated during the rapid propagation of the displacement fault causes the shock-hardened kamacite to recrystallise within a zone of finite thickness on either side of the surface of displacement. The visible width of this recrystallised zone varies from 1 to 20 μm according to the energy input at the particular fault.

We have examined a number of sections of Henbury 'shrapnel' in which relics of this 'mylonitised' kamacite are present at the outer fracture surfaces. To identify these relics of heat effect with shear mylonitisation, it is necessary to eliminate the possibility that the surface heating was produced by ablative friction during high-velocity flight through the atmosphere[2]. It is possible to do this since an ablative heat alteration zone[3] is composed of coarse ragged α_2 (martensitic) product that indicates reheating above 850 °C, whereas, by contrast, the effect observed at the surface of Henbury shrapnel is a fine-grained, recrystallised α (non-martensitic): the product of a much milder heating of shock-hardened kamacite.

The presence of shocked kamacite in Henbury shrapnel indicates that the fragmentation took place under conditions of shock loading with a shock pressure of at least 130 kbar. The shear faults seem to be superimposed upon the shock-hardened structure and, therefore, seem to have formed subsequently.

Once the fault has commenced to move on a selected surface it is likely to continue to do so because of the local generation of heat and the superplastic lubrication of the small kamacite grains in the mylonitised zone. The factors that cause a particular surface to be selected for shear faulting are, however, more difficult to specify, but, in general terms, they must arise from unbalanced shear forces as the projectile adjusts itself rapidly to the complex array of shock waves that accompany high-velocity impact with the Earth.

H. J. AXON
E. M. STEELE-PERKINS

Metallurgy Department,
The University,
Manchester M13 9PL, UK

1 Spencer, J. L., *Min. Mag.*, 23, 387 (1933).
2 Nininger, H. H., *Ward's nat. Sci. Bull*, 24, 21 (1950).
3 Brentnall, W. D., and Axon, H. J., *J. Iron Steel Inst.*, 200, 947 (1962).

Mechanism of the oxidation of ethylene by palladium(II)

THE oxidation of ethylene to acetaldehyde by aqueous palladium chloride forms the basis of the Wacker process. The kinetics and mechanism of this reaction have been extensively studied for ethylene and other olefins, and most authors assume that the formation of a *cis* hydroxoethylene-palladium complex is an essential step in the reaction[1–4]. We propose here a new mechanism by which the *trans* intermediate, $[C_2H_4Pd(OH)Cl_2]^-$, may directly yield the palladium σ-hydroxyalkyl species without the necessity of *trans–cis* isomerisation or the attachment of a fifth ligand.

It is widely accepted that the first steps of the reaction are the formation of a π-ethylene complex, $[C_2H_4PdCl_3]^-$ (Fig. 1a) the palladium analogue of Zeise's salt, followed by the aquation and dissociation of the *trans* aquo complex to give $trans[C_2H_4Pd(OH)Cl_2]^-$ (Fig. 1b). A *trans* to *cis* isomerisation is then assumed with the subsequent insertion of the ethylene into the palladium–hydroxide bond (a *cis*-migration rearrangement) giving a palladium σ-hydroxyalkyl molecule $[PdCl_2CH_2CH_2CH_2OH]^-$ (Fig. 1c) which yields acetaldehyde by a palladium assisted hydride shift. Little attention[5,6] has been focused on the isomerisation of $[C_2H_4Pd(OH)Cl_2]^-$ although it has been suggested[1,7] that a dihydroxo species is involved as an intermediate. An alternative view[4,8,9] is that a five-coordinate palladium(II) species is involved, thus avoiding the requirement of the *cis* configuration. The kinetic evidence for these latter proposals has been obtained at low chloride and acid concentrations and is somewhat unconvincing in the normal concentration range so that the isomerisation of $[C_2H_4Pd(OH)Cl_2]^-$ remains controversial.

We have already suggested[10] a model for catalysis by nickel (II) complexes, which was based upon the facilitation of certain molecular vibrations by the vibronic coupling of the highest filled molecular orbital with vacant molecular orbitals derived

Fig. 1 Compounds occurring during the oxidation of ethylene by palladium(II): *a*, π-ethylene complex; *b*, $trans[C_2H_4Pd(OH)Cl_2]^-$; *c*, the palladium σ-hydroxyalkyl molecule $[PdCl_2CH_2CH_2OH]^-$.
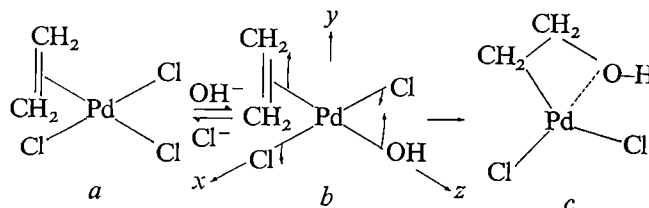
from ligand π-acceptor orbitals. Application of that model to the Wacker process is straightforward. The *trans* complex $[C_2H_4Pd(OH)Cl_2]^-$ is a planar molecule with C_{2v} molecular symmetry. The out-of-plane YMZ bending vibration of a MX_2YZ molecule of C_{2v} symmetry with the coordinate axes as shown in Fig. 1 has b_2 symmetry. If this vibration is facilitated in $[C_2H_4Pd(OH)Cl_2]^-$ then the C_2H_4 and OH^- ligands will move closer together as the molecule distorts towards a pseudo-tetrahedral structure allowing a ready path for the insertion of ethylene into the Pd–OH bond. Molecular orbital calculations are available[11,12] for Zeise's salt, $[C_2H_4PtCl_3]^-$, and because of the marked similarity of the electronic structures of $PtCl_4{}^{2-}$ and $PdCl_4{}^{2-}$ (ref. 13) we assume that the general qualitative features of the Zeise's salt calculations will be applicable to $[C_2H_4Pd(OH)Cl_2]^-$. Both calculations agree that the highest filled molecular orbital is of b_1 symmetry, although the exact nature of the orbital is debatable. Facilitation of the b_2 vibration may occur by the vibronic coupling mechanism if a vacant molecular orbital of a_2 symmetry ($b_1 \times b_2$) lies close enough to the filled b_1 orbital to lead at least to a reduction in the force constant of the b_1 vibration. Such an orbital is available: it is largely a non-bonding orbital of the metal complex derived from the molecular orbitals of the ethylene ligand. Symmetry considerations are thus consistent with the possible facilitation of the b_2 molecular vibration and the ethylene and hydroxide ligands may approach each other by the out-of-plane bending of the $[C_3H_4–Pd–OH]$ unit. Attack by the hydroxide on the π* orbitals of ethylene may occur either in an approximately end-on situation with the carbon atoms and the oxygen atom lying in the *zy* plane or, if the ethylene molecule is allowed to rotate about the palladium–ethylene axis, at the centre of the double bond. If, as suggested by Hartley[14], the palladium–olefin bonding is weaker than the platinum–olefin bond then attack on the largely vacant π* orbitals may be more likely leading to *cis*-insertion through a four membered metallocycle.

This mechanism provides an alternative to the *trans–cis* isomerisation which, together with rotation of the olefin, is a prerequisite for the *cis*-insertion reaction which is generally accepted at present.

ADRIAN W. PARKINS
ROGER C. SLADE

Chemistry Department,
Queen Elizabeth College,
London W8 7AH, UK

1 Jira, R., and Freiesleben, W., *Organomet. React.*, 3, 1 (1972).
2 Herbehold, M., *Metal π-Complexes, II*(2), 198 (Elsevier, Amsterdam, 1974)
3 Aguilo, A., *Adv. organomet. Chem.*, 5, 321 (1967).
4 Stern, E. W., *Transition Metals in Homogeneous Catalysis*, 93 (Dekker, New York, 1971).
5 Moiseev, I. I., Vargaftik, M. N., and Sirkin, Y. K., *Dokl. Akad Nauk. S.S S.R.*, 152, 773 (1963).
6 Henry, P. M., *J. Am. chem. Soc.*, 86, 3246 (1964)
7 Jira, R., Sedlmeier, J., and Smidt, J., *Annalen*, 693, 99 (1966)
8 Stern, E. W., *Catalysis Rev.*, 1, 73 (1971).
9 Okada, H., Noma, T., Katsuyama, Y., and Hashimoto, H., *Bull. chem. Soc. Japan*, 41, 1395 (1968).
10 Parkins, A. W., and Slade, R. C., *J. chem. Soc., Dalton* (in the press)
11 Kato, H., *Bull. chem. Soc. Japan*, 44, 348 (1971).
12 Rösch, N., Messmer, R. P., and Johnson, K. H., *J Am. chem. Soc.*, 96, 3855 (1974).
13 Messmer, R. P., Interrante, L. V, and Johnson, K. H., *J. Am. chem Soc.*, 96, 3847 (1974).
14 Hartley, F. R., *Chem. Rev.*, 69, 799 (1969).

Mechanisms of reactions at square planar metal centres

SQUARE-PLANAR platinum(II) complexes have in the past been found to react by associative mechanisms (see refs 1 and 2). This has been of some comfort to the proponents[3] of the '16 and 18 electron rules', which state that all stable transition metal complexes and intermediates in the reactions of such complexes should have either 16 or 18 electrons in the transition metal's valence shell. We point out here that though substitution reactions at platinum(II) occur by an associative route, reactions between ligands coordinated to platinum(II)—here called combination reactions—occur by a dissociative route that violates the '16 and 18 electron rules'.

Substitution reactions involve a trigonal bipyramidal transition state (reaction 1) and not a square pyramidal one, and in the trigonal bipyramidal transition state the leaving group

$$A_T - M - L + E \rightleftharpoons A_T - M \begin{smallmatrix} L \\ E \end{smallmatrix} \rightleftharpoons A_T - M - E + L \quad (1)$$
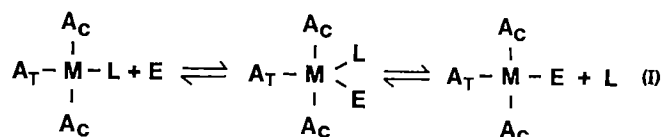
(L), the entering group (E) and the *trans*-ligand (A_T) all lie in the trigonal plane with angles of about 120° separating them.

Combination reactions, such as that involved in the insertion of olefins into platinum(II) hydride bonds to give platinum(II)-alkyls[4,5] and that involved in the insertion of carbon monoxide into platinum(II)-alkyl bonds to give acyl complexes[6,7], occur by a dissociative route (reaction 2) in which insertion occurs

$$L_2Pt(A)(B) \xrightarrow{+E} [L_2Pt(AB)] \rightarrow [L_2Pt(AB)E] \quad (2)$$
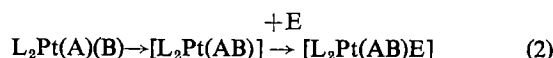
first to give an intermediate that is either 3-coordinate or 4-coordinate with the fourth coordination site occupied by a solvent molecule.

A similar mechanism has been suggested for the insertion of isocyanides into platinum(II)-alkyl and aryl bonds[8], and
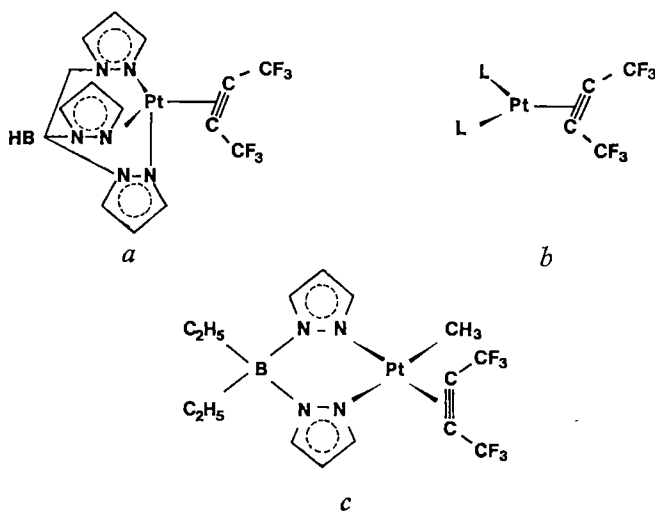
Fig. 1 Platinum(II) (methyl) acetylene complexes (see text).

it is likely that the insertion of olefins and acetylenes into platinum(II)-alkyl bonds follows the same route since an essential prerequisite for reaction is a 4-coordinate complex in which the alkyl and olefin groups are *cis* with respect to each other[9]. It is noteworthy in this connection that 5-coordinate complexes in which the alkyl and acetylene groups are *cis* with respect to each other are reluctant to undergo insertion. Thus the five-coordinate complexes in Fig. 1*a*, in which the Me–Pt–acetylene bond angle equals 88.5° (ref. 10), and in Fig. 1*b*, where L = PMe₂Ph, do not give insertion, whereas the five-coordinate complex in which L = AsMe₂Ph (Fig. 1*b*), and the four-coordinate complex shown in Fig. 1*c*, do give insertion.

Nucleophilic attack on olefins coordinated to platinum(II) and palladium(II) can occur either on the same side as the

metal (cis-attack; Fig. 2a) or on the side remote from the metal (trans-attack; Fig. 2b). Cis-attack will almost invariably involve prior coordination of the nucleophile to the metal. This coordination can, in principle, occur in two ways: first, by displacement of one of the ligands bound in the square-plane cis to the olefin giving a 4-coordinate intermediate; second, at one of the two axial sites giving a 5-coordinate intermediate. Analysis of the available evidence would suggest that cis-attack always involves the first of these two possible mechanisms. Thus cis-attack has been shown by stereo-chemical analysis of the products to occur when phenyl[12,13], carboxymethyl[12], chloride (sometimes)[14,15] and acetate (in the absence of chloride)[16] are the attacking nucleophiles. Kinetic investigations of the chloride[14] and acetate[16] reactions have shown that cis-attack involves reaction of a ligand originally bound to a site in the square-plane and does not involve an extra ligand coordinated to an axial site. Thus, cis-nucleophilic attack on olefins involves a mechanism essentially similar to that given in reaction 2 for the insertion type of combination reaction.
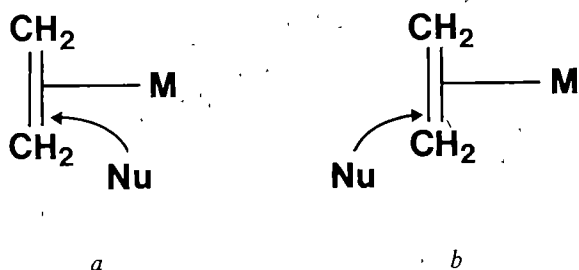
Fig. 2 Possible modes of nucleophilic attack on metal–olefin complexes: a, cis-attack, b, trans-attack.

Clearly, it is of interest to ask why substitution reactions involve a trigonal bipyramidal intermediate whereas combination reactions apparently involve reaction between two ligands bound in the square-plane. Though no definitive answer can be given two salient points can be made:

First, ligands bound in the square-plane lie closer to one another (~90° apart) than ligands in a trigonal bipyramidal intermediate where the incoming ligand and the ligand being attacked would presumably lie in the trigonal plane (~120° apart) by analogy with their geometry in substitution reactions. The fact that the methyl and acetylenic ligands in the trigonal bipyramidal complex (Fig. 1a) do not combine is of interest in this connection as they lie at almost 90° to each other. It is, however, possible that in this case reaction is not favoured electronically.

Second, coordination of ligands in the square-plane clearly involves a much stronger interaction between the ligand and the metal than does axial coordination. This facilitates combination reactions since coordination weakens the multiple bonds in unsaturated ligands such as carbon monoxide, olefins and acetylenes, because of the π-back donation of electron density to the ligand antibonding orbitals. That this bond-weakening is important is demonstrated by the stability of [(Et₂B(pz)₂)Pt(Me)(ac)] (Fig. 1c), where ac is PhC≡CMe or PhC≡CPh, compared with the rapid insertion that occurs when attempts are made to prepare the analogous complex with the strongly π-accepting acetylene, CF₃C≡CCF₃ (ref. 9).

It is far from clear why both substitution and combination reactions, on the basis of current experimental evidence, seem to spurn the square-pyramidal intermediate, particularly in view of the low energy barrier separating it from the trigonal bipyramidal configuration[17].

Nucleophilic attack on olefins coordinated to metal ions

occurs most readily when palladium(II) is the activating metal ion; other metal ions such as platinum(II) are much less active as promotors. A number of reasons have been advanced[18] to account for this.

First, palladium(II)–olefin complexes may be formed more rapidly than platinum(II)–olefin complexes.

Second, π-back donation from metal to olefin is likely to be less in the palladium(II)–olefin complex than in the platinum-(II)–olefin complexes because of the higher ionisation potentials of the valence shell electrons of palladium(II). Thus, the electron density around the olefinic double-bond would be less when it was coordinated to palladium(II) than to platinum(II), and so olefins coordinated to palladium(II) would be more susceptible to nucleophilic attack.

Third, the palladium(II)–olefin bond is weaker than the platinum(II)–olefin bond so that the activation energy of any rearrangement process that may be necessary during the course of the reaction will be lower for palladium(II)–olefin complexes than for platinum(II)–olefin complexes.

Fourth, palladium(II) can more readily expand its coordination sphere to accept a fifth and sixth ligand than can platinum(II). This would allow the incoming nucleophile to coordinate to palladium(II) before attacking the olefin, thus lowering the activation energy of the nucleophilic attack.

The fact that palladium(II)–olefin complexes such as [(C₂H₄)PdCl₂]₂ (ref. 19) and [Pd(CH₂=CH-CH₂NH₃)Cl₃] (ref. 20) are hydrolysed instantaneously, whereas their platinum(II) analogues[21,22] are hydrolysed, respectively, only on heating and not at all, suggests that though the first of those reasons may be important, it is by no means the limiting factor. Kinetic and stereochemical evidence suggests that the last reason is irrelevant because coordination of the attacking nucleophile to a fifth position out of the square-plane is abortive and does not precede the combination reaction between olefin and nucleophile.

We have recently carried out experiments to distinguish between the remaining reasons using methoxide as the nucleophile, as it is known to attack olefin complexes trans with respect to the metal[23], thus eliminating any complications caused by the greater preference of this nucleophile for coordination to one or other metal.

1. When platinum(II)–olefin complexes such as [(PPhEt₂)-PtCl₂(C₂H₄)] are treated with methoxide ion, the principle reactions involve displacement of ethylene from the platinum(II) complex to yield either [(PPhEt₂)PtCl₂]₂, or [(PPhEt₂)-PtCl₂(OMe)], which subsequently decomposes with the elimination of formaldehyde; nucleophilic attack on the olefin to yield methylvinylether is only a minor reaction[24].

2. Treatment of [(C₂H₄)MCl₂]₂ with methanolic sodium carbonate gave a 70% yield of CH₃CH(OMe)₂ and no detectable C₂H₄, CH₂=CHOMe or HCHO when M was palladium; but when M was platinum only C₂H₄ and HCHO (formed by decomposition of the initially formed platinum(II)–methoxy complex) were detectable. [CH₃CH(OMe)₂ is formed by nucleophilic attack by MeO⁻ at the β-carbon atom of -Pd-CH₂-CH₂OMe formed by nucleophilic attack on the co-ordinated ethylene.] Both experiments indicate that platinum(II)–olefin complexes readily undergo substitution reactions with methoxide ion, which suggests that the third reason outlined cannot be of major importance in determining the relatively low susceptibility of platinum(II)–olefin complexes to nucleophilic attack. It thus seems that palladium(II)–olefin complexes are highly susceptible to nucleophilic attack because the correct balance is achieved between the loss of electron density from the olefin pπ-bonding orbital through the olefin to the metal σ-bond, and the gain of electron density in the olefin pπ*-antibonding orbital through the metal to the olefin π-bond. Too much π-back donation prevents nucleophilic attack, as shown by platinum(II) and even more effectively by platinum(0), and too little π-back donation is equally effective in preventing nucleophilic attack as shown by silver(I).

F. R. HARTLEY

Department of Chemistry,
The University,
Southampton SO9 5NH, UK

J. J. PÉRIÉ

U.E.R. de Chimie Organique,
Université Paul Sabatier,
118 route de Narbonne,
31 Toulouse, France

1 Langford, C. H., and Gray, H. B , *Ligand Substitution Processes*, ch. 2 (Benjamin, New York, 1965).
2 Hartley, F. R., *The Chemistry of Platinum and Palladium*, ch. 11 (Applied Science, London, 1973).
3 Tolman, C. A., *Chem. Soc. Rev.*, 1, 337 (1972)
4 Clark, H. C., and Wong, C. S., *J. Am. chem Soc* , 96, 7213 (1974).
5 Clark, H. C., and Jablonski, C. R., *Inorg. Chem.*, 13, 2213 (1974)
6 Glyde, R. W., and Mawby, R. J., *Inorg. Chem* , 10, 854 (1971)
7 Wilson, C J., Green, M., and Mawby, R. J., *J. chem. Soc., Dalton*, 1293 (1974)
8 Treichel, P. M., and Wagner, K. P., *J. organometal Chem.*, 61, 415 (1973)
9 Clark, H. C., Jablonski, C. R., and Von Werner, K., *J. organometal. Chem.*, 82, C51 (1974).
10 Davies, B. W., and Payne, N. C., *Inorg Chem.*, 13, 1843 (1974)
11 Clark, H. C., and Puddephatt, R. J., *Inorg. Chem.*, 10, 18 (1971)
12 Heck, R. F., *J. Am. chem. Soc.*, 91, 6707 (1969)
13 Segnitz, A., Bailey, P M., and Maitlis, P. M., *Chem. Commun* , 698 (1973)
14 Henry, P. M., *J. org. Chem.*, 38, 1140 (1973).
15 Mann, B. E., Bailey, P. M., and Maitlis, P. M , *J. Am. chem Soc.*, 97, 1275 (1975)
16 Henry, P. M., *Chemical Society Annual Chemical Congress*, York, April 1975.
17 Berry, R. S., *J. chem. Phys.*, 32, 933 (1960)
18 Hartley, F. R , *Chem. Rev.*, 69, 799 (1969).
19 Smidt, J., *et al.*, *Angew Chem.*, 71, 176 (1959)
20 Hartley, F. R., and Wagner, J. L , *J. chem. Soc., Dalton*, 2282 (1972).
21 Anderson, J. S , *J. chem. Soc.*, 971 (1934)
22 Denning, R. G., and Venanzi, L. M., *J. chem. Soc.*, 3241 (1963).
23 Stille, J. K., James, D. E., and Hines, L. F., *J. Am. chem. Soc.*, 95, 5062 (1973).
24 Hartley, F. R., and Searle, G W., *J. organometal. Chem.*, 69, 315 (1974)

Search for isotope effects in heavy-ion diffusion in liquids

THERE is some controversy as to the existence of isotope effects in liquid diffusion in which isotopically labelled molecules diffuse in the same liquid medium. Friedman[1] has predicted from the standpoint of the correlation-function theory of diffusion that there should be little or no effect and certainly nothing approaching the inverse square mass relationship and this has been shown[2,3] to be true for organic liquids. Miller[4] claims, however, that uranyl ions diffusing in aqueous solution do show an isotope effect and also that heavier ions diffuse faster than lighter ions. He attributed the phenomenon to kinetic effects in the molecular diffusion process; in particular, to persistence of velocities and backscattering. The effects should apply generally to heavy ions diffusing in light

solvents, and in view of the reported separation factors the difference in diffusion coefficients may be expected to be measurable. I report here the results of experiments designed to test this supposition.

The heavy ions chosen for study were the pairs $^{125}I^-$, $^{131}I^-$ and $^{134}Cs^+$, $^{137}Cs^+$; in the former case the absolute mass difference is double that in the uranyl ion case. Both these ionic species are considered to be unhydrated so that their effective masses in aqueous solution are known fairly reliably. Diffusion coefficients for these ions were measured using the magnetically stirred diaphragm cell method[5]. The conditions which were varied in the course of these experiments were temperature, concentration and the type of diffusion. One set of tracer-diffusion results is shown in Table 1. In this first type of experiment, the solutions were uniform throughout the cell and there was a gradient in the trace radioactive components only.

Two further sets of binary diffusion experiments were also carried out. In these experiments there was a gradient in the salt concentration as well as in the radioactive component. In one case, 0.5 M KCl was allowed to diffuse into water and radioactive Cs^+ ions were added as tracer; in the other, 2.0 M KI diffused into water accompanied by radioactive I^- ions. It will be appreciated that the conditions of these two sets of experiments approach more closely those used by Miller[4] than do the tracer-diffusion set used in the earlier experiments. The results are shown in Table 2.

The results (Tables 1 and 2) indicate that within the 'average error' of about 0.2% there is no detectable isotope effect for these heavy ions under a variety of conditions. Each experiment in which both KCl and trace Cs^+ ions were diffusing into water could be internally calibrated since the diffusion coefficients of the system KCl-water were accurately known[5]. This refinement allows diffusion coefficients to be specified to within ±0.1% for this system.

The high separation factors reported in Miller's original paper[4], particularly those recorded when agar gel was used as the diffusion medium, were caused by transient diffusion effects and were not related directly to the steady state diffusion coefficients (L. Miller, personal communication). Miller[6] has

Table 2 Coefficients of isotopic ions in binary diffusion at 25 °C

Diffusing isotope	Medium	Mean $D \times 10^9$ ($m^2 s^{-1}$)	No. of expts	Average deviation from mean
$^{134}Cs^+$	0.5 KCl diffusing into water	1.871	3	±0.002
$^{137}Cs^+$		1.872	3	±0.003
$^{125}I^-$	2.0 M KI diffusing into water	2.062	3	±0.003
$^{131}I^-$		2.064	4	±0.002

Table 1 Tracer-diffusion coefficients of isotopic heavy ions in aqueous solution

Diffusing isotope	Medium	Temperature (°C)	Concentration (mol l^{-1})	Mean $D^*_i \times 10^9$ ($m^2 s^{-1}$)	No. of experiments	Average deviation from mean
$^{125}I^-$	KI	10	0.5	1.381	3	±0.004
$^{131}I^-$	KI	10	0.5	1.377	3	±0.006
$^{125}I^-$	KI	10	3.0	1.393	5	±0.002
$^{131}I^-$	KI	10	3.0	1.394	5	±0.002
$^{125}I^-$	KI	25	0.5	1.992	4	±0.005
$^{131}I^-$	KI	25	0.5	1.985	5	±0.007
$^{125}I^-$	KI	25	2.0	1.952	6	±0.004
$^{131}I^-$	KI	25	2.0	1.950	5	±0.004
$^{125}I^-$	KI	45	0.5	2.899	4	±0.010
$^{131}I^-$	KI	45	0.5	2.900	3	±0.003
^{134}Cs	KCl	25	0.5	1.935	5	±0.003
^{137}Cs	KCl	25	0.5	1.930	4	±0.002

derived from the transient separation data the difference between the diffusion mobilities of $^{235}UO_2^{2+}$ and $^{238}UO_2^{2+}$ ions and found it to be of the order of 0.25%. This latter result is compatible with those reported here because were a persistence mechanism responsible, it would affect less those ions which have a mass considerably below that of the uranyl ion. In any event, both studies show that isotopic effects in the diffusion of heavy ions in light solvents are either nonexistent or very small.

R. MILLS

Diffusion Research Unit,
Research School of Physical Sciences,
Australian National University,
Canberra, ACT Australia

1 Friedman, H. L., *Molecular Motions in Liquids* (edit. by Lascombe, J.), (Reidel, D , Dordrecht, 1974).
2 Allen, G. G., and Dunlop, P. J , *Phys. Rev. Lett.*, 30, 316 (1973).
3 Mills, R , *J. phys. Chem* , 79, 852 (1975).
4 Miller, L., *Nature*, 243, 32 (1973).
5 Mills, R., and Woolf, L. A , *The Diaphragm Cell* (Australian National University Press, Canberra, 1969).
6 Miller, L., *J. phys. Chem* (in the press).

Stereoillusion based on visual persistence

IF a moving vertical line is stroboscopically lit, it seems to undergo a multiplication, so that at any instant of time it is perceived as a series of lines, spread out in the direction of motion[1-3]. If the illumination going to the eye is reduced, the apparent multiplication increases[3], suggesting possibly that the effect is a function of sensory integration time or "persistence" as it is affected by adaptation state of the eye[2]. Since the adaptation conditions of the two eyes are substantially independent[4], it is interesting to ask what we should see if a stroboscopically moving target were viewed with a filter over one eye only, and the different numbers of apparent bars stereoscopically fused. In these conditions more bars should be signalled by the filtered than by the unfiltered eye, and it is not obvious what the fused percept will be.

To investigate this problem I used a haploscopic display in which the number of bars presented separately to the two eyes was controlled on an oscilloscope (CRO) display by a digital computer. One eye was presented with a single vertical bar (5° high) that moved in discrete steps of 0.67° every 40 ms. The bar can be thought of as a target moving at 1.0 Hz, peak-to-peak amplitude 9.5°, and stroboscopically illuminated at 25 Hz. The other eye was presented with two bars, one of which corresponded in spatial position with the bar in the first eye, and the second with the preceding position of that bar (Fig. 1). Fusion of the left and right eye displays was aided by means of a stereoscope positioned in front of the CRO. Observers were instructed not to "track" the apparently moving targets, and to aid steady fixation a vertical white (0.4 log foot lambert) line was provided in the optical plane of the CRO screen.

The appearance of the fused display was that of several bars travelling in an orbit around the fixation line. The number of lines was difficult to count accurately, but it was certainly often in excess of two. These bars moved as a unit, giving an impression of a picket fence in motion. There was a convincing impression of depth to the motion, the "fence" appearing to rotate in a clockwise orbit (as if viewed from above) with the extra bar in the left eye, and anticlockwise with the extra bar in the right eye. To demonstrate this, a random series of trials was programmed in which the extra bar appeared in the left and right eye, and observers (colleagues and undergraduates in the laboratory) pressed a button on each trial to indicate whether they saw clockwise or anticlockwise motion. The experimenter was not in the room. Responses of the observers very seldom varied from the rule that an extra left-eye bar produced clockwise motion, and an extra right-eye bar anticlockwise motion.

The direction of this effect is the same as that of the "Pulfrich"
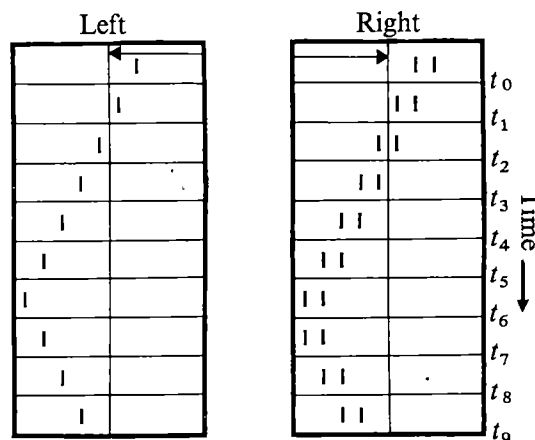
Fig. 1 Schematic representation of the display at successive times ($t_0, t_1 \ldots t_9$). In the actual display the left and right halves were presented separately to the left and right eyes and fused to give a single percept. In this particular representation the right eye has an extra bar, which is in a "trailing" position.

effect"[5], in which a continuously visible target seems to move in a clockwise orbit when viewed with a left-eye filter. If we recall that a reduction in illumination produces greater visual persistence, the facts are strongly suggestive of a common mechanism for the Pulfrich stereophenomenon and the new illusion reported here. To support this hypothesis, a neutral density filter in front of one eye was added to the display described above. With a suitably chosen filter (1.0 log units) in front of the left eye we expect depth to be enhanced when the extra bar is in the left eye, and reduced when it is in the right eye; conversely with the filter over the right eye. Observers were given a random mixture of trials with extra bar in the left and right eyes, first with the filter in front of one eye (40 trials) and then with the other eye filtered (40 trials). On each trial they were asked to say whether they saw clear depth (and in which direction) or whether the effect was weak. Since all observers had previously seen the strong effect without a filter, and since it was very quickly obvious to them that depth was absent or much reduced on a proportion of the trials, these judgments were accomplished without difficulty. The results, shown in Fig. 2, were as expected; clear depth was seen only when the Pulfrich, (filter) and persistence (extra bar) effects reinforced one another; when they were in opposition the depth effect was much reduced or absent.

Although these considerations favour a relationship between

Fig. 2 Mean data for three observers who saw displays like those illustrated in Fig. 1, but with a 1.0 log unit filter over one eye. When the filter was over the eye that had the extra bar, the percentage of trials on which the observer reported a clear depth effect (ordinate) was large. When the filter and the extra bar were in opposite eyes, there were many fewer confident depth judgments.
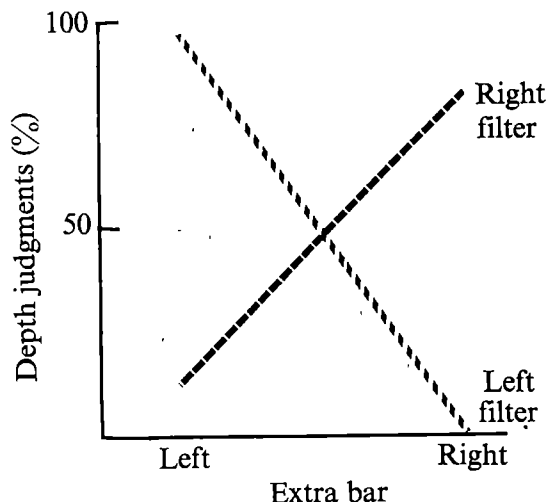
the Pulfrich effect and the effect reported here, the latter is not to be explained either by existing knowledge of static stereopsis, or by the conventional explanation of the Pulfrich effect itself. The most nearly related stereoscopic effect is that of "Panum's limiting case". in which a single bar in one eye is fused with a pair of bars in the other eye, to yield an impression of two bars at different distances. This is what will be seen if the two halves of Fig. 1 are fused in a stereoscope. In my display, however, there was not an impression of lines at different distances, but rather of a set of lines moving in the same orbit. It seems, then, that there is an important difference between the dynamic and static versions of the "limiting case". Similarly, Fertsch's classical explanation[5] of the Pulfrich effect in terms of latencies will not apply here. Fertsch proposed that the filtered eye signalled the actual position of the target at some later time than the unfiltered eye. In my experiments, however, the most advanced (leading) edge of the left and right-eye targets was identical, and differed only in one eye having a longer "trailing" edge. This reinforces other objections that have been advanced against the latency interpretation of the Pulfrich phenomenon[5]. The alternative explanation of filter-induced depth suggested by the present experiment is that the filter causes a trailing edge disparity between the two eyes, and that depth is determined, either by fusion of trailing edges of the signals, or after some spatial averaging has taken place. The possible importance of trailing edge information was realised long ago by Edmund[6]: "Of a movable object, the one eye particularly sees the posterior contour trailing behind, and that is the reason why one thinks that the image of the one eye really succeeds that of the other eye and thus, as Pulfrich's observation shows, receives a false stereoscopic impression."

I thank J. Mollon for discussions and Professor Zangwill for facilities.

MICHAEL MORGAN

The Psychological Laboratory,
University of Cambridge, Cambridge, UK

[1] Crawford, A., *Bull. Br. Psychol. Soc.*, 17, 2A–3A (1964).
[2] Allport, D. A , *Br. J. Psychol.*, 59, 395–406 (1968)
[3] Mollon, J , thesis, Univ Oxford.
[4] Crawford, B. H , *Proc. R. Soc.*, B128, 552 (1940)
[5] Morgan, M. J , and Thompson, P., *Perception* (in the press).
[6] Edmund, C., *Acta Ophthal* , 6, 414–424 (1928)

Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse

RANDOM X inactivation makes the female mammal a natural mosaic for clones of cells having either the maternally derived X (X^m) or paternally derived one (X^p) which is genetically inactive[1]. There are, however, instances in which inactivation is obviously not random[2–7]. Non-randomness was inferred from studies made on differentiated cells remote from early embryonic cells in which inactivation occurred. Thus it is not clear whether the randomness of the X inactivation process was influenced or whether cell selection occurring after random inactivation was responsible for the ultimate non-random appearance[4–9]. In an effort to determine the embryonic stage at which the X chromosome initiates differentiation in famale mouse embryos heterozygous for Cattanach's translocaton[10], we found that the mosaic composition was consistently biased in extraembryonic membranes, whereas it was not necessarily so in the embryonic body.

Cattanach's translocation comprises an insertion of a segment from chromosome 7 into an X (refs 11 and 12), making the X (X^t) the longest element in the cell[13]. In somatic cells from female heterozygotes, either X^t or the normal X (X^n) replicates asynchronously with the remaining chromosomes of the complement[14,15]. This is consistent with the c-variegation in their coats[10]. Since asynchronous replication of one X chromosome is a cytological manifestation of genetic inactivity[16,17], we used this criterion to ascertain the inactive X in the present study.

The allocyclic X chromosome of the mouse usually becomes late replicating by 8.5 d of gestation[18]. Before this stage the same chromosome initiates DNA replication later and finishes it earlier than the rest of the complement[18], which renders replication in the middle of the S phase more active in the former than in the latter. We expected, therefore, that the acridine orange (AO) fluorescence technique after the incorporation of 5-bromodeoxyuridine (BrdU)[19,20], for an appropriate period before fixation may allow not only the identification of every autosomal pair but also the distinguishing of the allocyclic X chromosome from the isocyclic one Preliminary tests, in fact, proved the technique satisfactory; the allocyclic X was identified in more than 80% of metaphases from 7.5-d embryos as the palest red (Fig. 1) or the brightest green element in the complement depending primarily upon the amount of incorporated BrdU[20].

The 6.5-d-old embryo consists of two germ layers with minimal mesodermal elements, and the proamniotic cavity and the exocoel join to form an elongated narrow lumen. The whole embryo, being small at this stage, provided just enough material for a single slide Either X^t or X^n showed differential staining (Fig. 2) in the heterozygous females from reciprocal crosses between the chromosomally normal (A/He) mice and the animals bearing a translocation. Surprisingly, however, the allocyclic X was predominantly paternal (Table 1). The proportion of cells with an allocyclic X^p closely agreed in heterozygous females from both types of crosses. This may imply that the decision of X inactivation was not necessarily random, otherwise the random decision was hidden by the overgrowth of cells having the isocyclic X^m. Before this point is made clear, it seems essential to know the distribution of two types of cells with either the allocyclic X^p or X^m in the embryo. If both cell types distribute unevenly, certain recognisably large areas consisting of a single cell type could be expected. We looked for such specific embryonic regions in embryos at 7.5 and 8.5 d of gestation.

The 7.5-d embryo, at late egg cylinder stage, is divided by the amnion into the embryonic and extraembryonic areas, which were studied separately. The mosaic constitution of both regions proved outstandingly different. The allocyclic X^p was found in less than 70% of cells from the embryonic area, whereas it was found in more than 90% of cells from the extraembryonic area. The decreased overall frequency of cells with the allocyclic X^p at this stage might be attributed to the increase of the embryonic elements relative to the extraembryonic ones in comparison with the earlier stage.

Mosaic composition was studied in five well-defined regions isolated from 8.5-d embryos, the anterior and posterior halves of the embryonic body, the allantois, the yolk sac, and the chorion. Cells with the allocyclic X^p were found to outnumber those with the allocyclic X^m in extraembryonic membranes, whereas the former was only slightly in excess of the latter in the embryonic body as such. The extreme instance was the chorion where the X^p seemed allocyclic in more than 95% of the constituent cells. Since intermingling of cells from different embryonic structures could not be rigorously excluded by the present dissection procedures, it is possible that the chorion is composed exclusively of cells in which X^m is active.

It therefore seems probable that X^p is mostly inactive in the chorion and the yolk sac of embryos heterozygous for Cattanach's translocation. Evidently, we are not dealing with the O^{hv} type mutation which makes the inactivation
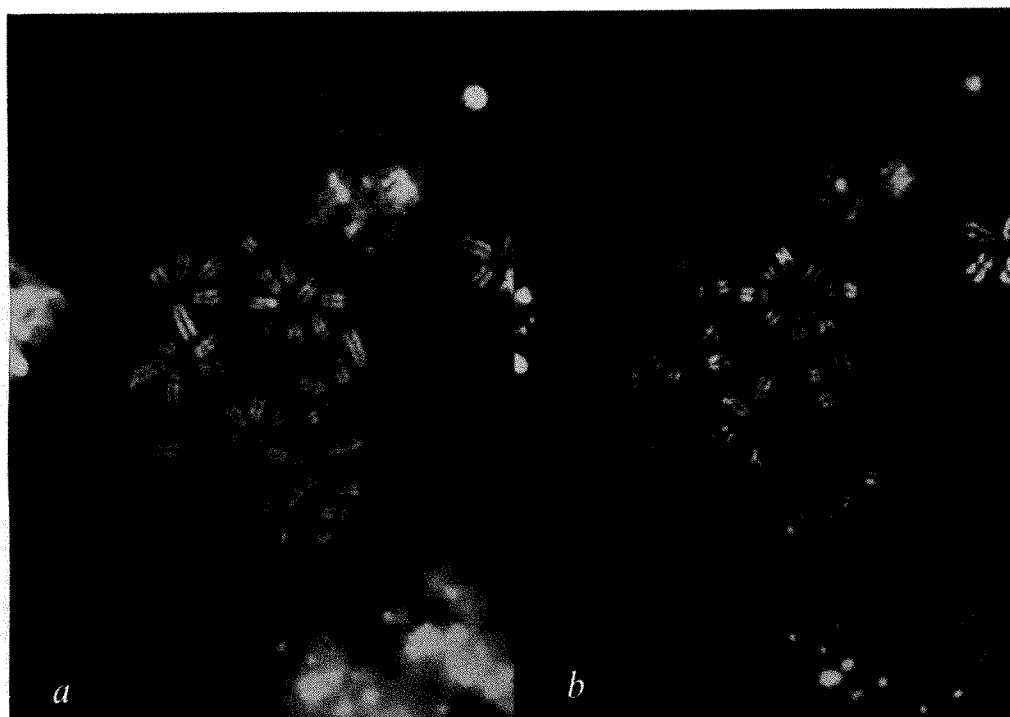
Fig. 1 Verification of the BrdU–AO technique in ascertaining the allocyclic X chromosome in the mouse (T6 heterozygote in this photograph). The brightest (allocyclic) X chromosome after QM technique (*a*) corresponds to the palest element in the complement after BrdU–AO technique (*b*). Embryos 7.5 d after fertilistion were taken out of the uterine cavity, incubated at 37 °C for 8 h in Eagle's medium supplemented with foetal calf serum (20%) and BrdU (100μg ml^{-1}). Colcemide (1 μg ml^{-1}) was added 2 h before collection. Air-dried slides were prepared accordingly to a modified method of Wroblewska and Dyban[21] as described by Takagi and Oshimura[22].

process nonrandom[23,24] since both cell types were nearly equally represented in the embryonic body as a whole. The slight excess of cells with an allocyclic X^p in the embryonic body seems to be at variance with the lower levels of the c-variegation when X^t is inherited from the father rather than from the mother[25]. Of particular interest are the mechanisms responsible for accomplishing divergent mosaic compositions in different regions of a single embryo. Our finding is apparently incompatible with random inactivation followed by random sampling of primordial cells for the extraembryonic structures or random inactivation after the divergence of the embryonic from the extraembryonic anlage, unless reversal of inactivation or extensive cell selection is advocated.

It is tempting to postulate that the preferential paternal X inactivation in the extraembryonic membranes is not restricted to Cattanach's translocation, but is common in the laboratory mouse and possibly in some other mam-

malian species. The eutherian type random X inactivation system of gene dosage compensation is considered to have evolved from the marsupial type paternal X inactivation system[26,27]. If so, does the peculiar feature represent a character directly inherited from the paternal X inactivation system? Or, has it been evolved after the establishment of random inactivation? In either case, it seems highly unlikely that the specific pattern of inactivation has been retained without any advantages for embryogenesis. Thus an assumption could be made that the active X^m in the extraembryonic membranes is favourable, though not indispensable to embryonic development. We did in fact find an exceptional case of a grossly retarded 8.5-d embryo whose yolk sac was composed predominantly of cells with an allocyclic X^p, but further studies are essential for testing the validity of our tentative hypothesis. Investigations along these lines might also prove significant in man. ·

Table 1 Parental influence on X-inactivation mosaic compoistion in 6.5–8.5-d-old female embryos carrying Cattanach's translocation

Area examined	Age (d)	Mating type	No. of embryos examined	No. of cells examined	% Cells with allocyclic X^p(range)
Whole embryo	6.5	I*	19	789	87(71–98)
		II†	22	928	86(77–100)
Embryonic region	7.5	I	11	980	66(53–82)
		II	16	1,729	68(48–80)
Extraembryonic region	7.5	I	11	677	91(83–100)
		II	16	1,094	92(79–100)
Anterior half of embryo	8.5	I	7	552	52(39–65)
		II	6	614	52(30–66)
Posterior half of embryo	8.5	I	7	1,056	55(43–69)
		II	7	774	57(44–70)
Allantois	8.5	I	6	44	77(67–100)
		II	5	179	66(55–83)
Yolk sac	8.5	I	5	745	90(88–98)
		II	7	378	89(52–98)
Chorion	8.5	I	7	444	97(96–100)
		II	7	482	98(95–100)

*X^n/X^n × X^t/Y
†X^t/X^n × X^n/Y

21 Wroblewska, J., and Dyban, A. P., *Stain Technol.*, **44**, 147–150 (1969).
22 Takagi, N., and Oshimura, M., *Expl Cell Res.*, **78**, 127–135 (1973).
23 Drews, U., Blecher, S. R., Owen, D. A., and Ohno, S., *Cell*, **1**, 3–8 (1974).
24 Ohno, S., Geller, L. N., and Kan, J., *Cell*, **1**, 175–184 (1974).
25 Cattanach, B. M., and Perez, J. N., *Genet. Res.*, **15**, 43–53 (1970).
26 Cooper, D. W., *Nature*, **230**, 292–294 (1971).
27 Brown, S. W., and Chandra, S. H., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 195–199 (1973).
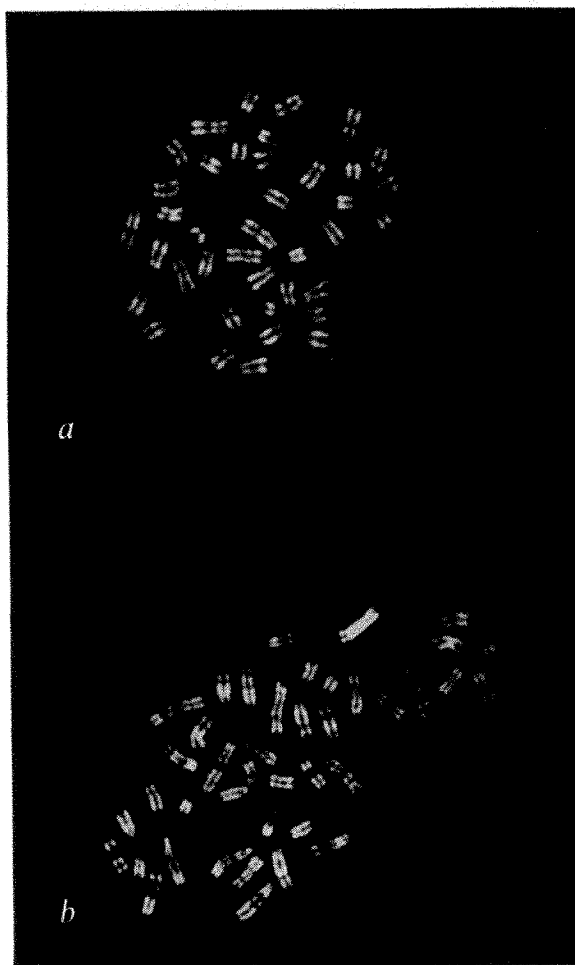
Fig. 2 Differential staining of the allocyclic X chromosome in cells from a 6.5-d-old female embryo heterozygous for Cattanach's translocation. The allocyclic X^t fluoresced faintly in *a*, whereas the allocyclic X^n fluoresced brightly in *b* depending, most probably, on the relative amount of incorporated BrdU.

NOBUO TAKAGI
MOTOMICHI SASAKI

Chromosome Research Unit,
Faculty of Science,
Hokkaido University,
Sapporo, Japan

1 Lyon, M. F., *Nature*, **190**, 372–373 (1961).
2 Lyon, M. F., *Phil. Trans. R. Soc. Lond.*, **B259**, 41–52 (1970).
3 Lyon, M. F., *Biol. Rev.*, **47**, 1–35 (1972).
4 Hamerton, J. L., Giannelli, F., Collins, F., Hallett, J., Fryer, A., and McGuire, V. M. *Nature*, **222**, 1277–1278 (1969).
5 Giannelli, F., and Hamerton, J. L., *Nature*, **232**, 315–319 (1971).
6 Hamerton, J. L., Richardson, B. J., Gee, P. A., Allen, W. R., and Short, R. V., *Nature*, **232**, 312–315 (1971).
7 Cohen, M. M., and Rattazzi, M. C., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 544–548 (1971).
8 Mukherjee, B. B., Mukherjee, A. B., and Mukherjee, A. B., *Nature*, **228**, 1321–1322 (1970).
9 Mukherjee, B. B., and Milet, R. G., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 37–39 (1972).
10 Cattanach, B. M., *Z. Vererbungsl.*, **92**, 165–182 (1961).
11 Francke, U., and Nesbitt, M. N., *Proc. natn. Acad. Sci. U.S.A.*, **68**, 2918–2920 (1971).
12 Kouri, R. E., Miller, D. A., Miller, O. J., Dev., V. G., Grewal, M. S., and Hutton, J. J., *Genetics*, **69**, 129–132 (1971).
13 Ohno, S., and Cattanach, B. M., *Cytogenetics*, **1**, 129–140 (1962).
14 Evans, H. J., Ford, C. E., Lyon, M. F., and Gray, J., *Nature*, **206** 900–903 (1965).
15 Nesbitt, M. N., and Gartler, S. M., *Cytogenetics*, **9**, 212–221 (1970).
16 Rattazzi, M. C., and Cohen, M. M., *Nature*, **237**, 393–395 (1972).
17 Ray, M., Gee, P. A., Richardson, B. J., and Hamerton, J. L., *Nature*, **237**, 396–397 (1972).
18 Takagi, N., *Expl Cell Res.*, **86**, 127–135 (1974).
19 Dutrillaux, B., Laurent, C., Couturier, J., and Lejeune, J., *C. r. hebd. Séanc. Acad. Sci. Paris*, **276**, 3179–3181 (1973).
20 Dutrillaux, B., and Fosse, A.-M,, *Annls Genet.*, **17**, 207–211 (1974).

Evidence derived from sister chromatid exchanges of restricted rejoining of chromatid subunits

SISTER chromatid exchanges (SCE) were first described by Taylor *et al.*[1] using autoradiographic techniques. After DNA replication in the presence of tritiated thymidine and subsequent replication in the absence of this radioisotope, two types of SCE were observed in colchicine-induced tetraploid cells. Twin exchanges represented events occurring in the second division cycle whereas single exchanges represented events occurring in the first division cycle.

If SCE rejoining occurs randomly between chromatid subunits, the ratio of twin to single SCE should theoretically be 1:10 (ref. 2). Alternatively, if SCE rejoining is restricted due to inherent polarity, this ratio should theoretically be 1:2 (ref. 2). Experimental support for these alternatives through the use of tritiated thymidine and autoradiography has been inconclusive, with ratios of twin to single SCE varying from 2.4:1 to 1:10 (refs 3 and 4). As an alternative method to examining tetraploid cells, the frequencies of SCE have been compared in metaphase cells one and two division cycles after tritiated thymidine labelling[5,6]. Again, results have been inconsistent, providing no clear evidence for either random or restricted rejoining of chromatid subunits. This variability is probably related to the use of tritiated thymidine, an agent known to induce SCE[7].

Recently, techniques have been developed which make possible the examination of SCE without radioactive labelling. These techniques are based on the differential staining of sister chromatids by fluorescent compounds[8,9] or Giesma[10] after a minimum of two division cycles in the presence of bromodeoxyuridine (BrdU). It was, therefore, decided to use these new techniques to re-examine whether SCE rejoining is random or restricted by comparing the frequency of SCE occurring in the first two division cycles with SCE occurring in the third division cycle.

Lymphocyte cultures from ten male subjects were incubated at 37 °C for 72 h in total darkness using culture media containing phytohaemagglutinin-M (Gibco) and 0.9 µg ml^{-1} BrdU. Colcemid (Gibco) was added to a final concentration of 0.1 µg ml^{-1} for the final 3 h of incubation. The cells were collected, fixed and slides prepared.

Metaphase chromosomes from cells having passed through one division cycle in the presence of BrdU exhibit no differential sister chromatid staining with acridine orange. Consequently, SCE cannot be detected (Fig. 1*a*). After two BrdU cell cycle divisions (Fig. 1*b*), SCE can be clearly observed since acridine orange stained chromatids unifilarly substituted with BrdU fluoresce more intensely than bifilarly substituted chromatids[9]. Mitotic cells after three BrdU cell cycle divisions can be easily distinguished from cells having undergone two replications in the presence of BrdU since only one-fourth of total sister chromatid length retains thymidine and fluoresces brightly (Fig. 1*c*).

In the examination of SCE frequency, previous investigators[5,12] have avoided the inclusion of SCE which seem to involve the centromeric region. Assumed difficulty in distinguishing centromeric SCE from chromosomal twists in this region was the basis for this exclusion. Centromeric SCE occurring in the first two division cycles, however, can be unequivocally determined in third division metaphase cells where they appear as chromosomes with only one arm brightly fluorescent (Fig. 1*c*). Examination of centromeric SCE fre-
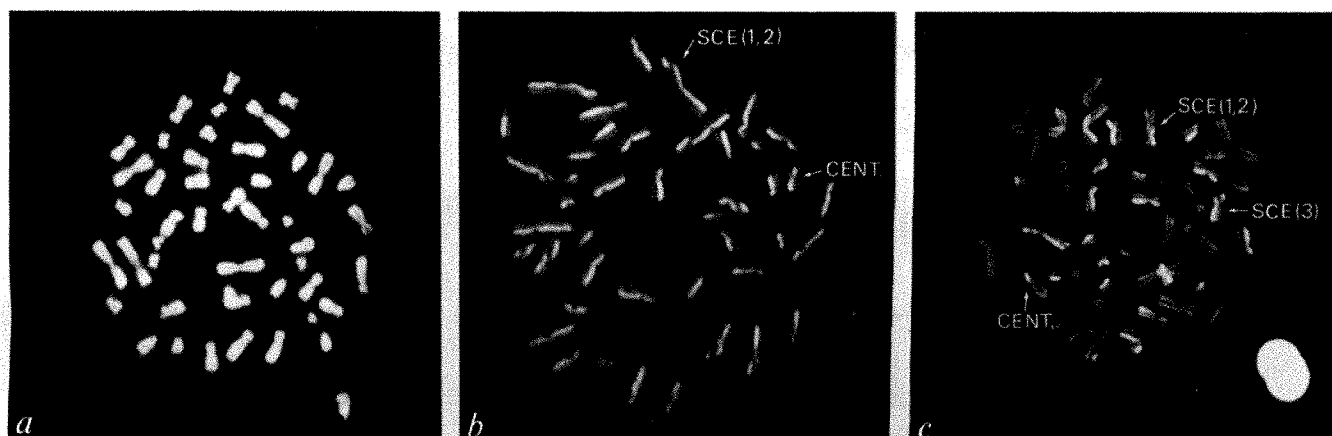
Fig. 1 Fluorescence photomicrographs of metaphase chromosomes. *a*, Cell after one division cycle in BrdU with all chromatids unifilarly substituted with BrdU and therefore no differential fluorescence; *b*, cell after two division cycles in BrdU with one-half of all sister chromatid regions bifilarly substituted with BrdU. These regions fluoresce less intensely than unifilarly substituted sister chromatid regions; *c*, cell after three division cycles in BrdU with only one-fourth of all chromatid regions still unifilarly substituted with BrdU. Arrows with (CENT.) denote centromeric SCE, arrows with (SCE (1, 2)) denote SCE which had occurred within the first two division cycles, arrows with (SCE (3)) denote SCE occurring in the third division cycle. Heparinised whole blood was cultured in Eagles MEM with Earle's salts, non-essential amino acids, 2 mM L-glutamine, penicillin (10,000 U ml⁻¹), streptomycin (10,000 μg ml⁻¹) (Gibco), and 10% heat-inactivated foetal bovine serum (Flow). Cultures were incubated at 37 °C for 72 h in total darkness in the presence of phytohaemagglutinin-M (Gibco) and 0.9 μg ml⁻¹ BrdU. Colcemid (Gibco) was added to a final concentration of 0.1 μg ml⁻¹ for the final 3 h of incubation. The cells were collected, fixed, and slides prepared[11]. Slides were stained for 5 min in 0.125 mg ml⁻¹ acridine orange[9] dissolved in phosphate buffer (*p*H 7.4), rinsed briefly with distilled water, incubated for 10 min in phosphate buffer (*p*H 7.4) and mounted in the same phosphate buffer. Chromosome fluorescence was observed in a Leitz Orthopian microscope equipped with epi-illumination from a 200 W mercury light source, BG12 and TK510 dichroic excitor filters, K515 and K510 suppressor filters. Photographs were taken using a ×54 objective.

quency in these cells showed them to occur as a relatively high proportion of all SCE. Consequently, in any comparison of SCE frequencies, it was decided that centromeric exchanges should be included. To assess our ability to distinguish centromeric SCE from chromosomal twists, we examined both second and third division metaphase cells for the proportion of centromeric SCE which occurred in the first two division cycles. Unequivocal centromeric SCE measured in third division metaphase cells accounted for 18% of all SCE occurring in the first two division cycles, whereas centromeric exchanges measured in second division metaphase cells accounted for 21% of all SCE. Therefore, it seems possible to distinguish true centromeric SCE occurring during any cell cycle from apparent chromosomal twists. These values for the proportion of SCE which occurs in the centromeric region are comparable with previously reported estimates[5,13].

The total SCE frequencies occurring in the first two division cycles are listed in Table 1. No significant difference was observed between measurements made in second or third division metaphase cells. Therefore, these data were combined and the ratio of SCE occurring in the first two division cycles to SCE occurring in the third division cycle was calculated. If SCE occur with equal frequency in each division cycle, the expected frequency of SCE occurring in the first two division cycles should be twice the frequency of SCE occurring in the third division cycle. Since only half of the SCE occurring in the third division cycle can be detected (half of the SCE occur in chromo-

somal regions completely substituted with BrdU), the expected ratio of the first two division cycle SCE to third division cycle SCE becomes 4 : 1. The derived ratio of 4.05 : 1 is clearly consistent with restricted rejoining of sister chromatid subunits. The ability to attain such a close approximation to the predicted ratio is most likely a result of the use of this new non-radioactive technique.

It has not been completely resolved whether the SCE observed with this technique are spontaneous or induced by BrdU. High concentrations of BrdU[10], or low concentrations of BrdU in the presence of light[14], have been shown to induce SCE. It is not clear whether BrdU at the low concentration we used in the absence of light induces SCE[10,15]. Irrespective of whether the observed SCE were induced or spontaneous, our results indicate that the observed SCE clearly involved both chromatid subunits with restrictions on rejoining due to subunit polarity. This does not exclude the possibility that in radiation or chemically treated systems, a proportion of induced SCE could be the result of exchange between single chromatid subunits.

Although our data strongly support a mononeme structure for eukaryotic chromosomes (that is, composed of one DNA duplex with chromatid subunits being single polynucleotide strands), it is also possible to devise a model by which SCE can occur with polarity restrictions on rejoining between sister chromatids composed of more than one DNA duplex. Such a polyneme model for SCE rejoining would, however, be several orders of magnitude more complex than a mononeme model.

Table 1 Mean SCE frequency

Division cycles in BrdU	(a) Frequency of SCE occurring in the first two division cycles (mean ± s.e.)	(b) Frequency of SCE occurring in the third division cycle (mean ± s.e.)	Derived ratio a/b	Expected* ratio a/b
2	10.50 ± 0.31 (165)	—	—	—
3	10.05 ± 0.33 (165)	2.54 ± 0.15 (165)	3.96	4.00
2 and 3	10.28 ± 0.23 (330)	2.54 ± 0.15 (165)	4.05	4.00

Thirty or more metaphase cells per individual (one-half second metaphase cells, one-half third metaphase cells) were optically examined by two observers for SCE frequency. Number of cells examined are shown in parentheses.
* Expected ratio based on restricted rejoining of chromatid subunits would be 2 : 1. Since only half of all SCE occurring in the third division cell cycle can be detected, however, the expected ratio becomes 4 : 1.
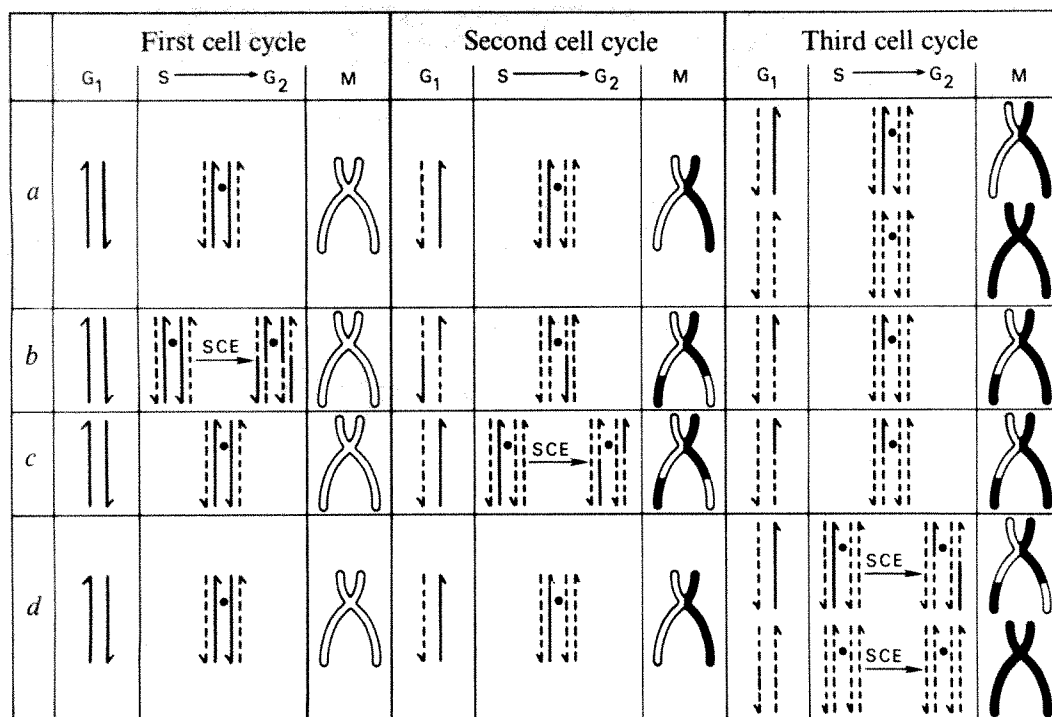
Fig. 2 Progression of cells through three division cycles in the presence of BrdU based on the unineme model of eukaryote chromosome structure. *a–d*, Appearance of chromosomes during three division cycles with: *a*, no SCE occurring; *b*, SCE occurring in the first division cycle; *c*, SCE occurring in the second division cycle; *d*, SCE occurring in the third division cycle. ———, Thymidine – – – –, BrdU-bearing DNA strand. Direction of arrow indicates polarity of chromatid subunits. Black chromosomal regions indicate regions which fluoresce less intensely with acridine orange. White chromosomal regions indicate regions which fluoresce more intensely with acridine orange.

Figure 2 is a schematic representation of the results of SCE occurring in each of the three successive replication cycles in the presence of BrdU based on the mononeme model of eukaryotic chromosome structure.

We thank Drs M. Bender and J. Rary and Mr P. Ciesla for support.

R. Tice

Department of Biology,
Johns Hopkins University,

J. Chaillet
E. L. Schneider

Laboratory of Cellular and
  Comparative Physiology,
Gerontology Research Center,
National Institute on Aging,
Bethesda
and *The Baltimore City Hospitals,*
Baltimore, Maryland.

1 Taylor, J. H., Woods, P. S., Hughes, W. L., *Proc. natn. Acad. Sci. U.S.A.*, **43**, 122–217 (1957).
2 Taylor, J. H., *Genetics*, **43**, 515–529 (1958).
3 Taylor, J. H., in *Molecular Genetics* (edit. by Taylor, J. H.), Part I, 85 (Academic, New York, 1963).
4 Sparoli, E., Gay, H., Kaufmann, B. P., *Proc. Third. Int. Congr. Radiat. Res.*, Cortina, 1966, 208 (1967).
5 Gibson, D. A., Prescott, D. M. *Expl Cell Res.*, **86**, 209–214 (1974).
6 Geard, C. R., *Mutat. Res.*, **23**, 67–78 (1974).
7 Gibson, D. A., Prescott, D. M., *Expl Cell Res.*, **74**, 397–402 (1972).
8 Latt, S. A., *J. Histochem. Cytochem.*, **22**, 478–491 (1974).
9 Kato, H., *Nature*, **251**, 70–72 (1974).
10 Wolff, S., Perry, P., *Chromosoma (Berl.)*, **48**, 341–353 (1974).
11 Hungerford, D. A., *Stain Technol.*, **40**, 333–338 (1965).
12 Chaganti, R. S. K., Schonberg, S., German, J., *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4508–4512 (1974).
13 Kim, My. A., *Humangenetik*, **25**, 179–188 (1974).
14 Ikushima, T., Wolff, S., *Expl Cell Res.*, **87** 15–19 (1974).

Genes for immunoglobulin heavy chain and serum prealbumin protein are linked in mouse

Several cases of allelic variation have been reported in the mouse that are presumably determined by genes for the variable region of the heavy chain of immunoglobulins[1–8]. These genes control qualitative and/or quantitative differences in the immune response to particular antigens, and exhibit close linkage to the cluster of genes (*Ig-1, Ig-2, Ig-3, Ig-4*) that determine the allotypic differences residing in the constant portion of the heavy chains of several immunoglobulin classes[9–11]. (No

recombinants have been observed among the latter in over 2,000 backcross mice.) Significantly, several presumptive recombinants have been recovered that seem to separate some variable region genes from the allotype loci, and some variable region genes from each other [1,12]. Outside genetic markers would be very valuable in identifying and interpreting these presumptive recombinants. Unfortunately, efforts to place the heavy chain allotype locus in the mouse linkage map have been unsuccessful [9,10,13]. We report here the discovery of close linkage between *Ig-1* and the serum prealbumin locus of the mouse.

The autosomal prealbumin locus (*Pre*) determines the presence or absence of a minor serum protein band that migrates ahead of the albumin band on electrophoresis[14]. Density of the band is hormone dependent, and is greatest in sexually mature males. The *Pre*ᵃ allele determines the presence of the band in both homozygotes and heterozygotes, whereas *Preᵒ/Preᵒ* homozygotes lack the prealbumin band. The absence of a detectable prealbumin band in *Preᵒ/Preᵒ* mice may reflect an altered mobility that coincides with the albumin fraction. The nature, function, and origin of the prealbumin protein is unknown[14,15]. The *Pre* locus has not been located in the linkage map[14].

Evidence favouring linkage of *Ig-1* and *Pre* came initially from observing the joint segregation of these loci in recombinant inbred (RI) lines. Three different sets of RI lines were studied. CXB lines developed by Bailey were derived by brother–sister mating from the F₂ generation of the cross of C57BL/6By and BALB/cBy (ref. 16). AKXL and BXD sets were similarly produced by Taylor from the crosses of AKR/J × C57L/J (ref. 17) and C57BL/6J × DBA/2J (ref. 18) respectively. It is clear that unlinked genes will be randomised in the F₂ generation, whereas closely linked genes will tend to become fixed in the same combinations as they entered the cross.

The results of typing the three sets of RI lines for *Ig-1* and *Pre* are presented in Table 1 with the genotypes of the progenitor strains. A preponderance of parental genotypes with respect to *Ig-1* and *Pre* was observed. Out of 54 independently derived RI lines, only 14 exhibited recombinant phenotypes ($P < 0.001$). If the loci were unlinked, a 1:1 ratio of parental to recombinant genotypes would be expected.

To confirm the linkage, sexually mature males from the C57L/J × (AKR/J × C57L/J)F₁ backcross were typed both for the presence of the prealbumin band and the AKR/J allotype. The numbers of mice in each of the four classes were as follows: *Pre*ᵃ/*Pre*ᵒ, *Ig-1*ᵈ/*Ig-1*ᵃ (51); *Pre*ᵒ/*Pre*ᵒ, *Ig-1*ᵃ/*Ig-1*ᵃ (54);

Pre^a/Pre^o, $Ig-1^a/Ig-1^a$ (9); Pre^o/Pre^o, $Ig-1^d/Ig-1^a$ (5). These results differ significantly from the expected 1:1:1:1 ratio in favour of linkage ($P<0.0001$). The estimated recombination fraction from the backcross data is 0.118 ± 0.034.

During the successive generations of inbreeding in the formation of an RI line, there are multiple opportunities for recombination between linked loci. The probability of fixing a recombinant genotype (R) is $4r/(1 + 6r)$, where r is the probability of recombination in a single meiosis[19]. Substituting 14/54 for R provides an estimate for r of 0.106 ± 0.040. The authors thank Dr E. L. Green for pointing out that r is equal to $R/(4-6R)$ and that the large sample variance of r estimated this way is $r(1 + 2r)(1 + 6r)^2/4N$, where N is the number of RI lines. The estimate obtained in this way is very similar to the estimate obtained in the backcross. The combined estimate of r is 0.114 ± 0.024. As this is a relatively short distance, we are probably justified in equating recombination frequency with map distance. The estimated map distance between Pre and $Ig-1$ is 11.4 ± 2.4 centimorgans (cM). Note that the estimate from the RI lines reflects the average recombination frequency in the two sexes, whereas the backcross data reflect recombination in males only.

Shreffler place the Pre^a allele of CBA/J on to the C57BL/10JSf genetic background by 10 backcrosses followed by brother–sister mating. The strain is designated B10.PRE. The allotype of the line proved to be that of the background strain ($Ig-1^b$) rather than that of the donor strain ($Ig-1^a$), indicating that a crossover in the $Ig-1–Pre$ region had occurred at some stage. This congenic strian may prove to be useful for crossing with Pre^o strains, when the C57BL background, the $Ig-1^b$ allele, and the Pre^a allele are all desired. It should also be useful in studies aimed at characterising the prealbumin protein as to site of synthesis, turnover rate and function.

The results of typing five $Ig-1$ congenic strains[1,10,11,20] in which the $Ig-1$ alleles from strains C57BL/Ka and AL/N (both Pre^o) were placed on the BALB/c (Pre^a) background are shown in Table 2. The BAB/14, C.B17, and C.B26 strains are all Pre^o type like the donor C57BL/Ka strain, indicating that no crossover has occurred between $Ig-1$ and Pre during the backcrossing and inbreeding process. This provides further confirmation of the linkage between $Ig-1$ and Pre. The C.AL9 and the C.AL20 strains are both Pre^a type unlike the donor AL/N strain, indicating that a crossover occurred between $Ig-1$ and Pre during their development. These results plus the finding of crossing over in the B10.PRE congenic strain, provide a basis for a third estimate of the recombination frequency between $Ig-1$ and Pre. The likelihood of obtaining recombination in the B10.PRE and C.AL series, without recombination in the C.B series is maximised by a recombination frequency of about 0.05, a value similar to the estimates obtained from the RI lines and backcross data. Note that the BAB/14 strain is an apparent recombinant between $Ig-1$ and the closely linked gene that determines an idiotype specific immune responsiveness to α-1,3 dextran[1]. This implies the gene order: $Pre–Ig-1–V_H–DEX$, unless a double crossover is postulated.

Extensive typing of the three sets of RI lines at other loci has not revealed the chromosomal location of Pre and $Ig-1$. The mapped loci with their respective chromosome numbers in parentheses, for which there exists at least partial typing in the CXB, AKXL, and BXD RI lines, are as follows: CXB: a (2), $Mup-1$ b $H-15$ $H-16$ $Gpd-1$ (4) $H(go)$ (5), $H-22$ $Gpi-1$ c Hbb $H-1$ (7), $Es-1$ $H-19$ (8), $Mod-1$ $H-7$ $Fv-2$ Bgs (9), Hba (11), and $H-2$ (17); AKXL: ln $Dip-1$ ald (1), $H-3$ Svp (2), $Mup-1$ b $Gpd-1$ (4), Gus (5), $Ly-2$ (6), c Hbb (7), $Es-1$ (8), $Thy-1$ $Fv-2$ (9), $Es-3$ (11), $H-2$ (17); BXD: $Id-1$ $Dip-1$ (1), Svp (2), $Mup-1$ b $Gpd-1$ $Fv-1$ (4), $Pgm-1$ (5), $Ly-2$ (6), $Gpi-1$ Hbb (7), $Es-1$ (8), d $Mod-1$ $Fv-2$ Bgs (9), $Es-3$ (11), and $H-2$ (17).

We have typed for prealbumin a number of inbred strains maintained by the Jackson Laboratory. Knowledge of this typing may be helpful in planning linkage studies of this region. To the list of Pre^a-bearing strains reported by Shreffler[14] (AKR/J, BALB/cJ, CBA/J, DBA/1J, DBA/2J, RF/J, and WB/Re),

Table 1 Segregation of $Ig-1$ and Pre among three sets of RI lines

RI lines (and progenitor strains)*	Genotypes†		No. of parental phase RI lines	No. of recombinant phase RI lines
	$Ig-1$	Pre		
(BALB/cBy), CXBG, CXBJ	a	a	2	
(C57BL/6By), CXBD, CXBE, CXBI	b	o	3	
CXBH, CXBK	b	a		2
(AKR/J), AKXL-8, 12, 17, 23, 25, 28, 37, 38	d	a	8	
(C57L/J), AKXL-1, 4, 6, 11 16, 19, 21	a	o	7	
AKXL-24	d	o		1
AKXL-13, 14, 18, 29, 36	a	a		5
(C57BL/6J), BXD-1, 2, 3, 5, 13, 14, 21, 22, 23, 29	b	o	10	
(DBA/2J), BXD-6, 11, 15, 16, 17, 20, 24, 27, 28, 30	c	a	10	
BXD-8, 9, 19, 25	b	a		4
BXD-12, 18	c	o		2
Totals				
Observed			40	14
Expected			27	27

$$\chi^2_1 = 12.5, P < 0.001$$

*CXB RI strains were sib-mated more than 30 generations before testing. AKXL and BXD RI lines were tested at a time when they were incompletely inbred ($F> 0.83$). Lines that were known to be segregating at either $Ig-1$ or Pre are not included. Certain deviations from continuous sib-mating occurred in AKXL lines 6, 11 and 16, the net effect being to slightly increase the probability of fixing C57L/J alleles. Several of the AKXL and BXD RI lines are now extinct, and other lines will likely become extinct.

†CXB RI strains were $Ig-1$ typed by Dr M. Potter[23]. AKXL and BXD RI lines were tested for the presence of the $Ig-1^d$ and $Ig-1^b$ alleles, respectively. Thus, from a d (or b) designation of RI lines for $Ig-1$, it should be interpreted that the $Ig-1^d$ (or $Ig-1^b$) allele was present in the line at the time it was tested. (Requisite antisera to test for the presence of $Ig-1^a$ and $Ig-1^c$ alleles were not available.) Allotyping was carried out using immunodiffusion with antisera made in BALB/c mice against conjugates of *Bacillus pertussis* and anti-pertussis antibodies made in NZB ($Ig-1^e$, cross reactive with $Ig-1^d$) and BAB/14 ($Ig-1^b$, otherwise congenic to BALB/c) mice, respectively. These allotyping sera precipitated only with immunoglobulins of appropriate strains[9-11].

Serum samples from two to six sexually mature males of each RI line were tested for the presence of prealbumin protein. Electrophoresis of fresh or frozen sera was carried out using a vertical gel electrophoresis cell (EC470, EC Apparatus, St Petersburg, Florida). A 5% Cyanogum 41 acrylamide gel was prepared as described by the manufacturer. Buffer was 0.09 M Tris, 0.09 M borate, 2.5 mM EDTA, pH 8.4 Electrophoresis of 10–20 μl serum samples was carried out for 2 h at a potential of 250 V. Gels were stained for 1h with 1% Amido Black in 5:5:1 methanol–water–acetic acid solution, and destained in the same solvent.

Table 2 Prealbumin types of several congenic strains in which different *Ig-1* alleles have been placed on the BALB/c (*Ig-1*ᵃ, *Pre*ᵃ) background by serial backcrossing*

Congenic strain	Donor strain	Generations of backcrossing	Crossover?
BAB/14 (*Ig-1*ᵇ, *Pre*ᵒ)	C57BL/Ka (*Ig-1*ᵇ, *Pre*ᵒ)	14	No
C.B17 (*Ig-1*ᵇ, *Pre*ᵒ)	C57BL/Ka (*Ig-1*ᵇ, *Pre*ᵒ)	17	No
C.B26 (*Ig-1*ᵇ, *Pre*ᵒ)	C57BL/Ka (*Ig-1*ᵇ, *Pre*ᵒ)	26	No
C.AL9 (*Ig-1*ᵈ, *Pre*ᵃ)	AL/N (*Ig-1*ᵈ, *Pre*ᵒ)	9	Yes
C.AL20 (*Ig-1*ᵈ, *Pre*ᵃ)	AL/N (*Ig-1*ᵈ, *Pre*ᵒ)	20	Yes

*The *Ig-1* congenic strains described here as well as the AL/N strain are maintained at the Institute for Cancer Research. The BAB/14 and C.B17 congenic strains were separated at the 13th backcross generation. Brother–sister inbreeding was instituted after one and four additional backcrosses in the BAB/14 and C.B17, respectively. The C.B26 congenic strain was separated from the C.B17 strain at the 17th generation. The C.B26 strain was backcrossed nine additional generations before inbreeding. The first 13 backcrosses were to the BALB/cAn strain. Subsequent backcrossing in the C.B17 and C.B26 strains was to the BALB/cAnNIcr subline. The C.AL9 and C.AL20 strains are related to each other in the same way that C.B17 and C.B26 are related. In the C.AL series all backcrosses were to the BALB/cAn strain. The designations used to represent these congenic strains varies between publications and do not follow the rules for standardised nomenclature. The designations used here are intended to be descriptive rather than definitive.

the following strains may be added: BDP/J, BUB/BnJ, CE/J, I/LnJ, LT/Re, NZB/BINJ, P/J, RIII/2J, SEA/GnJ, SEC/1ReJ, SJL/J, ST/bJ, and 129/J. To the list of *Pre*ᵒ-bearing strains reported by Shreffler (A/HeJ, A/J, C57BL/6J, C57BL/10J, C3H/HeJ, and WC/Re), the following strains may be added: AU/SsJ, CBA/CaJ, C3HeB/FeJ, C57BL/KsJ, C57BL/10Sn, C57BR/cdJ, C57L/J, C58/J, HRS/J, LG/J, LP/J, MA/J, MWT/Ww, SM/J, SWR/J, and WK/Re. The difference between the CBA/J and CBA/CaJ sublines explains a discrepancy in the reports of Shreffler[14] and Reuter *et al*[15]. The latter apparently typed a CBA subline related to CBA/CaJ. These two sublines exhibit multiple differences[21], indicating that they were either incompletely inbred at the time of separation or that contamination occurred. We have been unable to confirm Shreffler's *Pre*ᵃ typing of the WB/Re strain.

The *Pre* locus described by Shreffler and considered here should not be confused with another prealbumin electrophoretic variant designated *Pre-1* described by Claxton *et al*[22] that segregates independently of Shreffler's *Pre* locus. The close linkage of the *Pre-1* locus to the brown (*b*) locus in chromosome 4 suggests that it is identical to the *Mup-1* locus[23] that determines an electrophoretic variant in major urinary protein which is also detectable in serum[15].

The *Pre* locus has several merits as a marker for the *Ig-1* region; fresh or frozen serum is used for typing; there is a significant polymorphism among the commonly used inbred strains; typing is relatively easy, inexpensive and reliable. The weak and variable expression of the prealbumin gene in females and the inability to reliably distinguish between *Pre*ᵃ/*Pre*ᵃ and *Pre*ᵃ/*Pre*ᵒ mice limits the usefulness of this marker[14,15]. Both of these limitations may be removed by refinements in the typing procedure.

In man the heavy chain allotype locus (*Gm*) is linked to the protease inhibitor locus (*Pi*) with a map distance of about 25 centimorgans[24]. The *Pi* locus comprises an allelic series of electrophoretic variants of the serum glycoprotein α_1-antitrypsin[25]. Although murine α_1-anti-trypsin seems to share some chemicophysical characteristics with prealbumin[15,26], the former is electrophoretically monomorphic among inbred strains[26]. Further biochemical and biological characterisation of the prealbumin protein is in progress.

B. A. TAYLOR
D. W. BAILEY
M. CHERRY

The Jackson Laboratory
Bar Harbor, Maine 04609

R. RIBLET
M. WEIGERT

Institute for Cancer Research
Philadelphia, Pennsylvania 19111

1 Blomberg, B., Geckeler, W. R., and Weigert, M., *Science*, 177, 178–180 (1972).
2 Eichmann, K., *Eur. J. Immun.*, 2, 301–307 (1972).
3 Eichmann, K., and Berek, C., *Eur. J. Immun.*, 3, 599–601 (1973).
4 Sher, A., and Cohn, M., *Eur. J. Immun.*, 2, 319–326 (1972).
5 Pawlak, L. L., Mushinski, E. B., Nisonoff, A., and Potter, M., *J. exp. Med.*, 137, 22–31 (1973).
6 Imanishi, T., and Makela, O., *Eur. J. Immun.*, 3, 323–330 (1973).
7 Lieberman, R., Potter, M., Mushinski, E. B., Humphrey, W., and Rudikoff, S., *J. exp. Med.*, 139, 983–1001 (1974).
8 Weigert, M., Potter M., and Sachs, D., *Immunogenetics*, 1, 511–523 (1975).
9 Herzenberg, L. A., McDevitt, H. O., and Herzenberg, L. A., *A. Rev. Genet.*, 2, 209–244 (1968).
10 Potter, M., and Lieberman, R., *Adv. Immun.*, 7, 91–145 (1967).
11 Mage, R., Lieberman, R., Potter, M., and Terry, W. D., in *The Antigens* (edit. by Sela, M.), 229–374 (Academic, New York, 1973).
12 Eichmann, K., Tung, A. S., and Nisonoff, A., *Nature*, 250, 509–511 (1974).
13 Itakura, K., Hutton, J. J., Boyse, E. A., and Old, L. J., *Transplantation*, 13, 239–243 (1972).
14 Shreffler, D. C., *Genetics*, 49, 629–634 (1964).
15 Reuter, A. M., Kannes, F., Leonard, A., and Sassen, A., *Comp. Biochem. Physiol.*, 25, 921–928 (1968).
16 Bailey, D. W., *Transplantation*, 11, 325–328 (1971).
17 Taylor, B. A., Meier, H., and Myers, D. D., *Proc. natn. Acad. Sci. U.S.A.*, 68, 3190–3194 (1971).
18 Taylor, B. A., Heiniger, H. J., and Meier, H., *Proc. Soc. exp. Biol. Med.*, 143, 629–633 (1973).
19 Haldane, J. B. S., and Waddington, C. H., *Genetics*, 16, 357–374 (1931).
20 Bosma, M., and Bosma, G., *J. exp. Med.*, 139, 512–527 (1974).
21 Staats, J., *Cancer Res.*, 32, 1609–1646 (1972).
22 Claxton, L., Malling, H. V, and Walburg, H E., *Biochem. Genet.*, 11, 97–102 (1974).
23 Potter, M., *et al.*, *Genet. Res.*, 22, 325–328 (1973).
24 Gedde-Dahl, T., Fagerhol, M. K., Cook, P. J. L., and Noades, J., *Ann. hum. Genet.*, 35, 393–399 (1972).
25 Kueppers, F., *Am. J. hum. Genet.*, 25, 677–686 (1973).
26 Myerowitz, R. L., Chrambach, A., Rodbard, D., and Robbins, J. B., *Analyt. Biochem.*, 48, 394–409 (1972).

Chemotaxis of lymphoblasts

LYMPHOCYTES are motile cells whose movement *in vivo* seems to be directed, yet investigators over many years have repeatedly failed to show chemotaxis of lymphocytes *in vitro*. There have been some recent reports that lymphocytes migrate into micropore filters towards substances placed below the filter[1-3], but this migration was not found to be chemotactic[3]. It seemed to us that our and other people's failure to demonstrate lymphocyte chemotaxis might be because we were using the wrong population of cells. The lymphocytes which can be shown most clearly to migrate into inflammatory lesions *in vivo* are blast-transformed cells [4-6], and we therefore examined the migration in Boyden chambers of lymphoblasts from two sources; firstly cloned human lymphoblast cell lines maintained in continuous culture[7]; and secondly blast cells from the lymph nodes of CBA mice, either without deliberate sensitisation with antigen, or following exposure to the contact sensitising agent, oxazolone. Both human and mouse lymphoblasts were shown to migrate into filters towards chemoattractants. The nature of this migration is discussed below.

Locomotion and chemotaxis of blast-transformed lymphocytes were measured using the micropore filter technique as described previously[8]. For mouse lymphoblasts a filter of 8 μm pore size was used. Because the human blasts were larger, filters of 12 μm pore size (Sartorius, Göttingen, Germany) were used. The distance migrated by the leading front of cells was measured as described by Zigmond and Hirsch[9] after 3 h. At this time, the leading-front cells in a population of lymphoblasts migrating towards a strong chemoattractant have
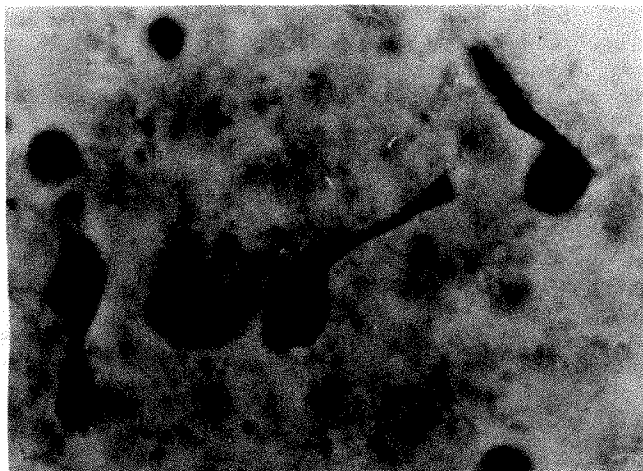
Fig. 1 Human lymphoblasts (line SMI_4) migrating in a micropore filter and showing locomotor morphology. The picture is take from above and the cells are at a depth of 60 μm in the filter. ($\times 900$)

migrated deeply into the filter but not reached the lower surface. This compares with 2 h for human blood monocytes migrating the same distance in the same filters. The cells did not require to be incubated in CO_2 (refs 1, 2) nor was prolonged incubation (18 h) necessary as has been suggested previously[1]. Table 1 shows the migration of lymphocytes from seven human lymphoblast cell lines in Gey's solution alone and in the presence, below the filter, of substances previously shown to be chemotactic for monocytes or neutrophils. The cell lines varied considerably in their random unstimulated migration and in their response to chemoattractants. Cells from all of the lines migrated further in the presence of endotoxin-activated plasma than in its absence and most of them also showed an enhanced response to casein. The migrating cells showed a typical locomotor morphology with a broadened leading edge and a narrow tail which occasionally became very elongated (Fig. 1).

To test whether the migration was chemotactic, experiments were set up in which the absolute concentration and the gradient of the chemoattractant were varied above and below the filter. The influence of chemoattractants on either rate of locomotion or on its orientation has been distinguished in this way[9]. Table 2 shows that the rate of locomotion of lymphocytes in the absence of a gradient (figures along the diagonal from upper left to lower right) varies with chemoattractant concentration, that is the cells show chemokinesis under these conditions. Furthermore, lymphocytes migrating in a positive gradient penetrated deeper into the filter than would be expected on the basis of concentration-dependent random migration alone; conversely,

lymphocytes migrating in a negative gradient did not penetrate as deeply as would be expected by random migration alone. These results support the conclusion that the locomotor response of human lymphoblasts to activated plasma depends not only on the absolute concentration of the plasma but also on the concentration gradient and is, therefore, chemotactic. Similar results were obtained with three other human lymphoblast lines. Table 2b shows the effects of colchicine on the migration of the same cells. Colchicine enhances concentration-dependent random migration in the absence of a gradient (figures on the diagonal), but colchicine-treated cells lose the capacity to detect and respond to a gradient. Similar results were obtained with vinblastine (10^{-5} M). These results resemble our unpublished data on the effects of colchicine and vinblastine on neutrophil and monocyte locomotion. They support the hypothesis[10-12] that the integrity of microtubules is required for the orientation of locomotion of these cell types but not for locomotion itself.

The lymphocytes from the auricular nodes of unsensitised mice are mostly small cells and the type of filter used was not suitable for the study of the migration of such cells. The small number of blast cells present in suspensions from normal mouse lymph nodes were, however, shown to migrate into filters and gave a dose response to chemoattractants which resembled that of human lymphoblasts (Fig. 2). Cell suspensions made from the draining nodes of mice 72 h after contact sensitisation by painting the ears with oxazolone contained many more blast cells (30%) than suspensions from unsensitised
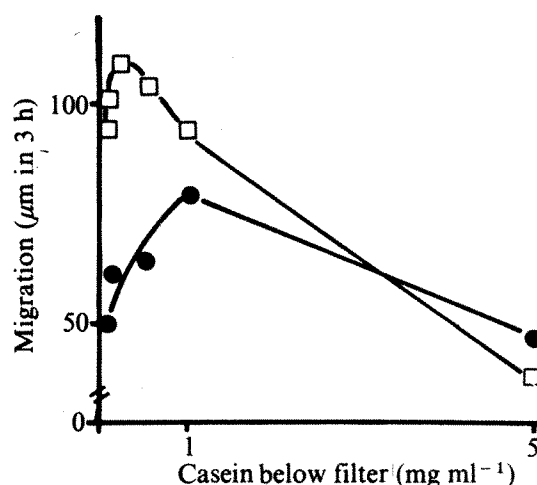
Fig. 2 Migration towards varying doses of casein of lymphoblasts from normal mouse auricular lymph nodes (●) and auricular lymph nodes 72 h after sensitisation with oxazolone (□)

Table 1 Migration of cultured monoclonal human lymphoblasts

Cell line	Source (refs 7, 13, 14, 15)	Ig release into supernatant (ref. 15)	Migration (μm in 3 h) towards		
			Gey's	Endotoxin-activated human plasma (10%)	Casein 1 mg ml⁻¹
SMI_4	Healthy cord blood	ML	28	98	75
BAR_1 1448a	Healthy XXY	GMKL	18	38	57
ANA_2	Healthy cord blood	GMKL	45	83	—
F_{89} 9489	Subacute lymphatic leukaemia	no Ig	21	78	30
FAL_1 8440	Healthy cord blood	No Ig	43	72	38
JIM_1	Healthy adult blood	AMK	90	120	97
ORI_1	Healthy adult blood	G	17	44	46

The cell lines were obtained from Dr Judith Evans, MRC Clinical and Population Genetics Unit, Western General Hospital, Edinburgh. They were established and maintained in tissue culture as described by Steel[7]. For studies of locomotion they were maintained in Eagle's medium +10% foetal calf serum and HEPES buffer (3% of a molar solution) changed every 4 d. They were incubated in 5% CO_2 at 37 °C until required for use when they were washed twice in serum-free Gey's solution before being placed in chemotaxis chambers at 2×10^6 ml⁻¹. Normal human plasma was activated by incubation at 37 °C for 30 min with *Shigella flexneri* lipopolysaccharide.

Table 2 The effect of varying concentration gradient and absolute concentration of chemoattractant on locomotion of human lymphoblasts (line F_{89} 9489)

Lymphocyte migration (μm in 3 h). Mean of 5 fields in each of 2 filters

a, No colchicine

% Endotoxin-activated plasma below filter

	0	0.5	3.5	6.5	9.5
0	18				
0.5		26	72(52)	81(52)	82(58)
3.5		29(52)	79	85(83)	90(82)
6.5		62(80)	67(83)	87	93(81)
9.5		53(82)	76(84)	75(81)	75

b, Colchicine (10^{-4}M) both sides of filter

	0	0.5	3.5	6.5	9.5
0	21				
0.5		83	90(91)	96(94)	87(95)
3.5		92(91)	99	93(98)	93(98)
6.5		89(97)	92(98)	97	94(98)
9.5		94(97)	90(98)	92(98)	98

Figures along the diagonal from upper left to lower right indicate distance migrated (μm) in increasing concentrations of the activated plasma but in the absence of a concentration gradient. Above the diagonal, the cells were moving in a positive gradient, below, in a negative one. The figures in brackets are estimates of what migration would have been in each of the tests assuming that the cells detected only the absolute concentration of the chemoattractant but not the gradient. They were calculated after Zigmond and Hirsch[9]. P for gradient effect on migration of cells without colchicine < 0.0005: with colchicine, > 0.1.

nodes (3%) and these cells were much more motile than those from the unsensitised nodes. They usually, but not always, showed considerable random motility in the absence of any chemoattractant (Fig. 2). This was also true of the blast cells from such nodes which did not adhere to nylon wool. Their response to chemoattractants was variable. Typically those which showed high unstimulated motility showed little further increase in motility when placed in the presence of a chemoattractant (Fig. 2). Experiments on the lines of Table 2 were done to test whether oxazolone-stimulated blast cells show a chemotactic response. In two out of three such experiments—the third gave an equivocal result—lymphoblasts from oxazolone-sensitised lymph nodes failed to show evidence of chemotaxis when tested under varying gradient conditions as in Table 2. These cells also consistently showed little or no change in the rate or the direction of their migration after treatment with colchicine. This might suggest that the blast cell population obtained after contact sensitisation shows a form of locomotion which is not directed or restrained by the action of microtubules.

These preliminary results suggest that populations of blast-transformed lymphocytes vary considerably in their locomotor response to chemoattractants, more so indeed than neutrophil or macrophage populations. The majority of cultured human lymphoblastoid B cell lines show low unstimulated motility, move faster in the presence of a chemoattractant, and show variations in their migration when the concentration gradient is varied which are consistent with a chemotactic response. This should be confirmed by time-lapse studies of the migration of single cells and the demonstration of oriented movement in a gradient by direct observation. Oxazolone-sensitised mouse lymphoblasts, however, show considerable unstimulated migration, and although they also respond to chemoattractants, this response does not seem to be chemotactic. It remains to be established whether these variations in locomotor behaviour reflect fundamental differences between B and T cell populations. It seems likely that the migration of lymphoblasts which we have observed *in vitro* is related to the migration of similar cells *in vivo* into sites of inflammation, especially since activated plasma is similar to attractants found *in vivo* in inflammatory tissue. Whether other examples of apparently directed lymphocyte migration *in vivo* are mediated by chemoattractants remains to be seen.

R. J. RUSSELL
P. C. WILKINSON
F. SLESS
DELPHINE M. V. PARROTT

Department of Bacteriology and Immunology,
University of Glasgow,
Western Infirmary,
Glasgow G11 6NT, UK

[1] Ward, P. A., Offen, C. D., and Montgomery, J. R., *Fedn Proc.*, **30**, 1721-1724 (1971).
[2] Higuchi, Y., Honda, M., and Hayashi, H., *Cell. Immun.*, **15**, 100-108 (1975).
[3] Schreiner, G. F., and Unanue, E. R., *J. Immun.*, **114**, 809-814 (1975).
[4] Asherson, G. L., Allwood, G. G., and Mayhew, B., *Immunology*, **25**, 485-494 (1973).
[5] Moore, A. R., and Hall, J. G., *Cell. Immun.*, **8**, 112-119 (1973).
[6] McGregor, D. D., and Logie, P. S., *J. exp. Med.*, **139**, 1415-1430 (1974).
[7] Steel, C. M., *J. natn. Cancer Inst.*, **48**, 623-628 (1972).
[8] Wilkinson, P. C., *Chemotaxis and Inflammation* (Churchill-Livingstone, Edinburgh, 1974).
[9] Zigmond, S. H., and Hirsch, J. G., *J. exp. Med.*, **137**, 387-410 (1973).
[10] Bhisey, A. N., and Freed, J. J., *Expl Cell Res.*, **64**, 419-429 (1971).
[11] Allison, A. C., Davis, P., and de Petris, S., *Nature new Biol.*, **232**, 153-155 (1971).
[12] Bandmann, V., Rydgren, L., and Norberg, B., *Expl Cell Res.*, **88**, 63-73 (1974).
[13] Jensen, E. J., Korol, W., Dittmar, S. L., and Medrek, T. J., *J. natn. Cancer Inst.*, **39**, 745-754 (1967).
[14] Steel, C. M., *J. natn. Cancer Inst.*, **47**, 1193-1201 (1971).
[15] Evans, J., Steel, C. M., and Arthur, E., *Cell*, **3**, 153-158 (1974).

Ultrastructural evidence for ectoglycosyltransferase systems

CONSIDERABLE biochemical evidence suggests that glycosyltransferases, in addition to being present in the Golgi apparatus[1], are also located on the outer surface of the plasma membrane of a variety of normal and malignant cell types[2-9]. The enzymes catalyse the transfer of specific monosaccharide units from nucleotide sugar donors to appropriate glycoprotein or glycolipid acceptors. Presumably those in the Golgi play a major role in the biosynthesis of glycoproteins and glycolipids[1] while those at the plasma membrane (ectoglycosyltransferases) have been implicated in mechanisms for cell to cell adhesion, recognition and communication[2-7]; cell surface repair[8] and for binding circulating oligosaccharides or glycoconjugates[9]. Although the biochemical evidence for the existence of ectoglycosyltransferases is compelling, it is not definitive. In this report, we use electron microscope (EM) autoradiography to demonstrate the plasma membrane localisation of a N-acetylneuraminic acid ectoenzyme system (ectosialyltransferase, EC 2.4.99.1; the enzyme together with cell surface acceptor molecules constitute the ectoenzyme system[8]). The results provide the first ultrastructural evidence for the presence of such a glycosyltransferase system at a cell surface.

Murine leukaemic L-1210 cells, shown previously in our laboratory[8] by biochemical methodology to possess galactosyl- and sialyltransferase activities at their external cell surface were again used in this study. The cells were obtained from the ascites fluid of DBA/2 female mice, washed twice in ice-cold phosphate buffered saline (PBS, Dulbecco's), pH 7.4,

adjusted to 10^8 cells ml^{-1} and incubated in PBS containing 10 µM CMP-^{3}H-N-acetylneuraminic acid (CMP-^{3}H-NANA) for 15 or 60 min at 37 °C. It was assumed that under these conditions whole nucleotide monosaccharides do not enter the cell. When the cells were incubated with CMP-^{3}H-NANA in the presence of a 100-fold higher concentration of free unlabelled N-acetylneuraminic acid, N-acetylmannosamine, mannosamine or mannose (all competitors of possible labelled degradation products of CMP-^{3}H-NANA), there was no decrease in incorporation levels (Table 1, experiments 1 and 2). Apparently, extracellular degradation, enzymic[10] or otherwise, of CMP-^{3}H-NANA with subsequent uptake and incorporation of labelled products was not responsible for the cell labelling. In addition, paper chromatography[8] of the cell medium following incubation with CMP-^{3}H-NANA revealed that less than 1% of the total radioactivity was present as free ^{3}H-NANA. It was concluded, on the basis of the above, that incorporation of ^{3}H-NANA from CMP-^{3}H-NANA into glycoconjugate must be taking place at the cell surface. We attempted to confirm this autoradiographically.

To attain sufficient incorporation levels of ^{3}H-NANA for EM autoradiography to be feasible, L-1210 cells were treated with *Vibrio cholerae* neuraminidase (VCN) for

Fig. 1 Electron microscope autoradiographs of labelled L-1210 cells incubated 15 min with CMP-^{3}H-NANA (*a*) or ^{3}H-galactose (*b*). The autoradiographic methods of Salpeter and Bachmann[11] were applied as described previously[12]. Ultramicrotome sections (1,000 Å thick) were mounted on collodionised slides, stained with 2% uranyl acetate, carbon coated and overlaid with a monolayer of Ilford L-4 emulsion. The preparations were exposed 25–55 d, developed in D-19 (Kodak) for 2 min at 24 °C and photographed with a Siemens Elmiskop 101. Note that for cells labelled with CMP-^{3}H-NANA, the grains lie mainly over the plasma membrane or within the range of radiation scatter from it while for cells labelled with ^{3}H-galactose, the grains are concentrated over the Golgi apparatus ($\times 2,772$).

15 min before incubation with CMP-^{3}H-NANA. The procedure presumably increases the available endogenous acceptor sites at the external cell surface for ectosialyltransferase and provides a sixfold increase in incorporation of label without affecting cell viability (Table 1, experiments 1 and 2). After a 15-min incubation in 10 µM CMP-^{3}H-NANA, cells were washed thoroughly in PBS, pelleted, fixed in 3% buffered glutaraldehyde (*p*H 7.0), postfixed in 1% osmium tetroxide, dehydrated and embedded in Spurr medium. Sections of the cells were processed for EM autoradiography[11,12]. Only the monosaccharide incorporated into glycoprotein, and not that present in the cell as free sugar, nucleotide sugar or glycolipid, is retained after this tissue processing[13,14].

EM autoradiographs of L-1210 cells incubated with CMP-^{3}H-NANA under the above conditions consistently showed grains over the plasma membrane of the cell (Fig. 1*a*). When for comparison, cells were labelled under the same conditions for 15 min with 10 µM ^{3}H-galactose (New England Nuclear, 0.6 Ci mmol^{-1}) in PBS and examined, the grains were concentrated mainly over the Golgi apparatus (Fig. 1*b*). A comparison of the relative grain densities over several cellular compartments (Table 2) clearly defines the organelles where incorporation of either label is taking place. The mode of incorporation of ^{3}H-NANA directly on to external plasma membrane glycoconjugate from its nucleotide monosaccharide represents a definite departure from that of free monosaccharides[13,14] which are ordinarily incorporated by means

Table 1 *In vitro* incorporation of N-acetylneuraminic acid from CMP-^{3}H-NANA by murine leukaemic L-1210 cells

Experiment	Conditions	^{3}H-NANA incorporation (d.p.m. per 10^7 cells)	
		15-min incubation	60-min incubation
1	Cells	3,104	18,109
	Cells+NANA*	3,228	19,423
2	VCN-treated cells†	19,661	94,167
	VCN-treated cells+NANA	22,248	121,071
3	Cells labelled (21,100 d.p.m. per 10^7 cells) as in experiment 2 and post-treated 15 min with VCN‡	2,069	—
4	VCN-treated cells preincubated§		
	0 min in PBS	19,380	91,062
	30 min in PBS	18,800	92,101
	60 min in PBS	19,100	90,381

5×10^7 L-1210 cells were suspended in 0.5 ml PBS containing 10 µM CMP-^{3}H-NANA (New England Nuclear, 2.33 Ci mmol^{-1}). The cell suspensions were incubated for 15 or 60 min at 37 °C in a Dubnoff metabolic shaker. Viability remained $> 96\%$ throughout the experiments as measured by Trypan blue dye exclusion. Incubations were terminated by the addition of 2 ml of 1% phosphotungstic acid in 0.5 N HCl. Acid-insoluble material was collected by centrifugation (500*g* for 5 min) followed by two washes with 10% trichloroacetic acid. The insoluble material was washed once with ethanol:ether (2:1) and dissolved in 0.2 ml 1 N NaOH. This material was neutralised with 1 N HCl and the radioactivity determined by scintillation counting as previously described[8]. The incubations were performed in duplicate on at least three different occasions. The data are expressed as the means of those experiments.
*During the incubation in CMP-^{3}H-NANA, 1 mM NANA was included in the medium as a competitor of possible CMP-^{3}H-NANA degradation products. N-acetyl-mannosamine, mannosamine and mannose were also separately tested with results similar to those shown for NANA.
†5×10^7 washed cells were treated with 10 units of *Vibrio cholerae* neuraminidase (Behring Diagnostic Company, EC 3.2.1.18) in 0.5 ml PBS for 15 min at 37 °C. The cells were washed three times in 2 ml PBS before incubation with CMP-^{3}H-NANA.
‡Cells labelled as in experiment 2 (−NANA, 15 min) were washed three times in 2 ml PBS, resuspended in 0.5 ml PBS containing 10 units of VCN and incubated for 15 min at 37 °C. Acid-insoluble radioactivity was determined as above.
§5×10^7 washed VCN-treated cells were suspended in PBS and preincubated for 0, 30 or 60 min at 37 °C. CMP-^{3}H-NANA was added to the same medium to a final concentration of 10 µM and the cells were incubated for 15 or 60 min at 37 °C.

of multiple activation steps in the Golgi apparatus as exemplified here with galactose, a known[13] glycoconjugate marker.

To demonstrate that the radioactivity associated with the cells was actually ³H-NANA, labelled cells were exposed to VCN for 15 min. The enzyme released 90% of the bound radioactivity from the cells (Table 1, experiment 3). Paper chromatography[8] revealed that 86% of the radioactivity recovered into the medium was ³H-NANA and 14% CMP-³H-NANA. This latter may be a result of incomplete washings after the labelling period.

It is conceivable that the transfer of ³H-NANA on to the cell surface might have been catalysed by free enzyme in the incubation medium incidentally secreted by Golgi vesicles[15] or otherwise released into the medium by cell surface shedding. To test this possibility, washed VCN-treated cells were preincubated 0, 30 or 60 min in PBS before the addition of CMP-³H-NANA to the cell medium, to allow enzyme accumulating in the cell medium during the preincubation period to then catalyse higher levels of incorporation in the 30 or 60-min preincubation samples. But in fact, the 30 and 60-min preincubation samples resulted in no additional incorporation during subsequent incubation in the presence of CMP-³H-NANA (Table 1, experiment 4). This indicates that labelling of the cell surface is not a result of a sialyltransferase being released into the medium from the cells before or during the labelling period. Further, there was no detectable transfer of ³H-NANA on to cells treated at 56 °C for 60 min (to inactivate endogenous cell surface enzymes) by the medium from a 15-min preincubation with viable cells.

The ultrastructural data reported here, together with those obtained biochemically, are evidence for the presence of an ectosialyltransferase system at the surface of L-1210 cells. We note that the bound radiation demonstrated at this location does not represent the enzyme itself, but macromolecular acceptors that have been glycosylated by the sialyltransferase. As with most EM cytochemical techniques for localising enzymes (with exceptions such as the labelled-inhibitor methodology[16]), it is the product of the enzyme reaction that is demonstrated and not the enzyme itself. The precise role of such enzymes or their acceptors is uncertain. Roseman[2] has postulated that batteries of different glycosyltransferases on the plasma membrane bind to acceptor molecules on opposing cell surfaces in a lock and key fashion to establish cell recognition and/or early adhesion. This postulate has recently gained much experimental support from several biochemical studies[4-7]. A more direct assessment of the role of ectoglycosyltransferases (or the macromolecules they glycosylate) in cell behaviour may now be achieved through the application of the methodology used in this study. In addition, the enzyme(s) may serve as a selective means for physiologically introducing an isotopic label on to specific cell surface glycoconjugates.

CARL W. PORTER
RALPH J. BERNACKI

Department of Experimental Therapeutics,
Grace Cancer Drug Center,
Roswell Park Memorial Institute,
New York State Department of Health,
666 Elm Street,
Buffalo, New York 14263

1 Schachter, H., Jabbal, I., Hudgin, R. L., Pinteric, L., McGuire, E. J., and Roseman, S., *J. biol. Chem.*, **245**, 1090–1100 (1970).
2 Roseman, S., *Chem. Phys. Lipids*, **5**, 270–297 (1970).
3 Bosmann, H. B., *Biochem. biophys. Res. Commun.*, **48**, 523–529 (1972).
4 Bosmann, H. B., Bieber, G. F., Brown, A. E., Case, K. R., Gersten, D. M., Kimmerer, T. W., and Lione, A., *Nature*, **246**, 487–489 (1973).
5 McLean, R. J., and Bosmann, H. B., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 310–313 (1972).
6 Roth, S., and White, D., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 485–489 (1972).
7 Roth, S., McGuire, E. J., and Roseman, S., *J. Cell Biol.*, **51**, 536–547 (1971).
8 Bernacki, R. J., *J. Cell Physiol.*, **83**, 457–466 (1974).
9 Pricer, W. E., and Ashwell, G., *J. biol. Chem.*, **246**, 4825–4833 (1971).
10 Kean, E. L., and Bighouse, K. J., *J. biol. Chem.*, **249**, 7813–7823 (1974).
11 Salpeter, M. M., and Bachmann, L., *J. Cell Biol.*, **22**, 469–477 (1964).
12 Porter, C. W., Chiu, T. H., Wieckowski, J., and Barnard, E. A., *Nature new Biol.*, **241**, 3–7 (1973).
13 Neutra, M., and Leblond, C. P., *J. Cell Biol.*, **30**, 137–150 (1966).
14 Bennett, G., Leblond, C. P., and Haddad, A., *J. Cell Biol.*, **60**, 258–284 (1974).
15 Hughes, R. C., *Prog. Biophys. molec. Biol.*, **26**, 189–268 (1973).
16 Barnard, E. A., and Rogers, A. W., *Ann. N.Y. Acad. Sci.*, **144**, 584–612 (1967).

Serological identification of HL-A-linked human 'Ia-type' antigens

THE Ir genetic region of the mouse which lies between the H-2K and H-2D loci that control the original serologically detectable H-2 specificities, has also been shown to contain genes controlling a new set of serological specificities called Ia for immune associated. Antisera raised by cross immunisation between H-2 recombinant strains have been shown to recognise the products of several genes within the Ir region (reviewed in ref. 1). Unlike the H-2K and H-2D antigens the Ia antigens have a tissue distribution that often does not include T lymphocytes.

By analogy with the mouse, human Ia antigens have been defined by mixed lymphocyte culture (MLC) inhibition and by serological tests on HL-A identical unrelated individuals[2-5]. We describe here some new approaches to the ready identification of sera against Ia-type antigens in man which make use of lymphoblastoid cell lines derived from B cells[6].

Extra reactions of HL-A-typing sera with lymphoid lines have been described[7,8]. A similar increase in reactivity has been noted when typing chronic lymphocytic leukaemia (CLL) cells derived from B cells[9-11]. Using HL-A-negative variants of lymphoid lines some of these extra reactions have been shown to be caused by antigens other than HL-A[12].

The fact that all these anomalous reactions are with cells presumed to be derived from B lymphocytes suggested that they may be directed against polymorphic antigens specific to B lymphocytes. Furthermore, the suggestion[12] that antibodies to these antigens tended to occur in HL-A-typing sera containing antibodies to particular serologically-defined LA and four locus antigens, indicated an association between the production of these antibodies and particular HL-A antibodies. This could most easily be explained by linkage disequilibrium[13] between the genes determining the corresponding antigens which, together with the evidence for B cell specificity, suggested that the anomalous reactions with lymphoid lines and CLL cells may be directed against human equivalents of the mouse H-2-linked Ia antigens.

In the first systematic screen for such reactions we used the B-cell-derived lymphoid lines T51 (ref. 14) and DAUDI (ref. 15), and our standard fluorochromatic cytotoxicity assay. DAUDI does not express the usual serologically determined

---

Table 2 Grain density distributions for cells incubated with CMP-³H-NANA or ³H-galactose

Compartment	Relative grain density	
	CMP-³H-NANA	³H-galactose
Plasma membrane*	100	16
Cytoplasm	11	14
Osmiophilic vacuoles	0	14
Golgi apparatus	7	100
Nucleus	2	7
Nucleolus	0	5

Photographs of labelled cells were taken at random and enlarged to a final magnification of ×10,000. Grains and points from a superimposed grid (1 point = 9 μm³ at ×10,000) were scored according to the cellular compartment they occupied. Grains per μm² minus background (0.2 grains per 100 μm²) were determined for each compartment and then normalised so that the compartment with the highest grain density was set equal to 100 and all other densities for that label adjusted proportionally. Relative grain densities for cells labelled with CMP-³H-NANA are based on 230 cells, 780 grains and 1,300 grid points. Those for ³H-galactose are based on 175 cells, 850 grains and 1,200 grid points.

*Includes all grains and points within 3,000 Å of the actual membrane to allow for radiation scatter[12].

Donor*	P1530B % +ve cells	P2604B % +ve cells
HB	24%	21%
EB	26%	20%
DB	14%	ND
EP	20%	ND
JB	<1%‡	ND
WB	20%	22%
JGB	26%	27%
LJ	25%	26%
JG	2%†	ND
GP†	<2%‡	ND
JJ	29%	ND

*Lymphocytes separated from defibrinated blood on a Ficol–Triosil gradient and stored frozen in liquid nitrogen.
†Cells of serum donor confirming the polymorphism.
‡Results giving <5% positive cells were considered to be negative for a given serum.
ND, Not determined.

HL-A antigens of the LA and four loci, nor does it have $\beta_2$microglobulin (β_2m)[16]. Any sera reacting with DAUDI are therefore presumed to have antibodies against Ia-type antigens. Out of 108 pregnancy sera previously characterised as having lymphocytotoxic HL-A antibodies when screened on a cell panel covering all the known LA and four loci antigens, ten were found which reacted with DAUDI or gave reactions with T51 that could not be explained by its HL-A type.

Serum P1530B, a weakly cytotoxic anti-HL-A9 antibody, was selected for further investigation using an indirect immunofluorescence assay[16]. Table 1 suggests that P1530B was reacting preferentially with B cells of some, but not all donors. As further evidence for B cell specificity, the percentage of lymphocytes reacting with P1530B was compared with the percentage reacting with a fluorescent anti-human IgM antibody. The differences were not significant in five cases tested. Furthermore, labelling with anti-human IgM antibody and P1530B failed to increase the percentage of fluorescing cells. B lymphocytes separated from peripheral lymphocytes by 'E' sheep red cell rosetting also showed an increased percentage of positive cells with P1530B, the rosetted T cells being totally unreactive. The positive reactions could not be removed by absorption with HL-A9-positive platelets (4×10^9 ml^{-1}) which did remove the weak cytotoxic HL-A9 antibody.

Two lines of evidence indicate that the P1530B reaction with B lymphocytes is directed against an antigen determined by a gene in the HL-A region, thus supporting the analogy with the mouse Ia antigens. The first is that out of a series of six somatic cell hybrids derived from a cross between the mouse L cell derivative A9 and DAUDI (refs 16 and 17) the only positive clone by immunofluorescence was also the only one containing human chromosome 6, which carries the HL-A region[18,19]. Subclones of this and other similar hybrids are now being studied to confirm this assignment. The second is that the results of a study of the immunofluorescence reactions of

P1530B on peripheral blood lymphocytes of parents and offspring from three families, were consistent with linkage between the HL-A system and the gene determining the antigen recognised by P1530B.

Screening for serum reactions similar to P1530B was extended by testing a panel of HL-A2 antisera for their cytotoxic activity on DAUDI, 8866 and its HL-A2-negative variant 8866 1-2 (ref. 12). Reactions with 8866 1-2 or DAUDI, are indicative of Ia-type reactivity. Out of 13 such sera tested, all of which reacted with 8866, two (5005 and 7155A, Table 2) also reacted with 8866 1-2 and DAUDI, whereas the remaining 11 were negative on these lines. This approach to detecting Ia-type activities, however, is limited by the need for a number of well-characterised HL-A-negative lymphoid line variants. It did

Sera	Origin	BRI8	BRI8 stripped
4025C (Pinquette)	NIH Walford	4	1
7086A (EYR/8573)	Sheffield	3	1
4245A (7234)	van Rood	3	1/2
4253A (Butterfield)	Rodey	3	4
1087B (Di Maggio)	Bodmer/Payne	2	1
P3098B	Bodmer (Oxford)	4	1
P3229B	Bodmer (Oxford)	4	1
7155 (CIC111544)	Sheffield	3	4
5005 (CLB11)	Engelfriet	4	4
5221 (Jochum)	E. Cohen	4	1/2
3002X (Caminiti)	Bodmer/Payne	4	1
Rabbit anti-β_2m	Dakopatts	4	1/2

Scores: 4, very strong positive; 3, strong positive; 2, weak, but definitely positive; 1/2, slight reaction, doubtful positive; 1, negative. Stripping of β_2m was carried out essentially according to Bernoco et al.[19] using rabbit anti-human β_2m (Dakopatts, Denmark) followed by a purified horse anti-rabbit IgG antibody, which was absorbed with an equal volume of human red blood cells and 10^8 DAUDI cells per ml serum to remove nonspecific effects. Stripped cells were suspended in a medium containing 10 mM sodium azide and then used in a cytotoxicity assay.

however, suggest another way of screening sera for Ia-type activity, namely to remove HL-A from lymphoid lines or from peripheral blood lymphocytes by 'stripping'[19] with anti-β_2m and anti-immunoglobulin sera.

The removal of β_2m from the surface of peripheral blood lymphocytes in this way also removes the associated allogeneic chain of the HL-A molecule and thus results in a complete loss of HL-A antigenicity. As DAUDI seems to have Ia-type antigens on its cell surface but not β_2m, at least some of these Ia antigens are presumably not normally associated with β_2m on the cell surface and so would not be removed from normal cells by stripping of the β_2m with specific antisera. In this way, therefore, we can turn any line effectively into a completely HL-A-negative variant and then screen for other Ia-type activities not associated with β_2m.

Reactions of the line Bri8 (HL-A1, 2, 8, 13) before and after

Cells	HL-A type	Sera									
		P1048B	P1530B**	P2242B	P2318B	P2377B	P2482B	P2604B	P2762B	P2902B	P2961B
Bri8*	1, 2, 8, 13	1	2	3	4	2	1	3	1	3	4
T51†	1, 2, 8, 27	4	4	4	4	3	4	1	1	3	3
8866‡	2, 3, 7—	1	4	1	4	3	3	4	1	4	1
MICH§	2, 32, 15, 17	1	2	1	1	1	4	4	4	1	4
BRI7‖	2, 9, 12, 17	3	1	4	3	1	1	4	1	1	4
DAUDI¶	—	2	4	2	3	4	3	2	4	4	4

Sera (including P1530B) were selected in the course of routine screening of pregnancy sera for HL-A activity because of their reactions with DAUDI. Scoring system as in Table 1.
*Cells from Searle Diagnostics; †see text; ‡cell line initiated by Dr G. Moore; §cells from Searle Diagnostics; ‖cells from Searle Diagnostics; ¶ see text; **P1530B was also found to be positive on the lymphoid lines Ba-13, Des3 and Sha, and negative on the lymphoid lines OR-1 and Bec 11.

stripping with essentially the same set of HL-A2 antisera used on 8866 and its variant, are shown in Table 2. The reactions of HL-A2-positive peripheral lymphocytes with this set of antisera could be completely removed by β_2m stripping. The same two sera (5005 and 7155) that reacted with the HL-A-negative variant of 8866 (and with DAUDI) also reacted with β_2m stripped Bri8. The only other serum to react in this way was 4253A, all the remaining sera, including as a control the anti-β_2m, were negative. These results validate the use of stripped lymphoid line cells for the detection of Ia-type activity. Reaction of 4253A with stripped Bri8 but not 8866 1-2 or DAUDI indicates some complexity in the Ia polymorphism. This is confirmed by more extensive typing of several of the lymphoid lines after β_2m stripping with a total of 20 antisera that have been shown to exhibit these Ia-type activities. A sample of these reactions is shown in Table 3. There is a complex pattern of reactions which is reminiscent of the early days of HL-A typing with unselected pregnancy sera, before the serologically detectable antigens had been properly defined. It seems likely that these initial Ia antisera will be complex mixtures of possibly cross reacting antibodies and further work is now being carried out to define the specificities properly.

Very striking associations between HL-A antigens and specific diseases have been established[20,21]. There are, however, a number of weak associations with the serologically detectable antigens which may simply reflect weak linkage disequilibrium between the genes in the HL-A region controlling disease susceptibility and those controlling the antigens of LA, 4 and AJ series. MLC typing in multiple sclerosis patients[22] has demonstrated that in such cases alleles at other loci in the region may show much stronger associations and so lead to both a better definition of the HL-A association and possibly its cause. Serological Ia typing will undoubtedly prove to be a powerful tool in future disease association studies.

While this paper was being written, Winchester *et al.*[23] reported a somewhat analogous study on B lymphocyte specific human antigens also using lymphoid lines. Details of our investigations will be published elsewhere.

We thank C. Barnstable and J. Brown for discussions, Professor H. Harris for providing the DAUDI xA9 hybrids, Dr A. Williams for the purified horse anti-rabbit IgG antibody, Drs W. Sly, M. Steel and G. Moore for lymphoid lines and many colleagues for exchanges of HL-A typing reagents. This work was supported in part by a grant from the MRC. P.G. and E.J. acknowledge predoctoral studentships from the MRC.

E. A. Jones
P. N. Goodfellow
J. G. Bodmer
W. F. Bodmer

Genetics Laboratory,
Department of Biochemistry,
University of Oxford, Oxford, UK

1 Schreffler, D. C., and David, C. S., *Adv. Immun.*, 20, 125–195 (1975).
2 van Leeuwen, A., van Schmit, H. R. E., and van Rood, J. J., *Transplant. Proc.*, V, 1539–1541 (1973).
3 Legrand, L., and Dausset, J., *Transplant. Proc.*, VII, No. 1, 5–8 (1975).
4 Ceppellini, R., *et al.*, *Transplant. Proc.*, III, No. 1 58-70 (1971).
5 van Leeuwen, A., Schmit, H. R. E., and van Rood, J. J., *Transplant. Proc.*, V, No. 4, 1539 (1973).
6 Patengale, P. K., Smith, R. W., and Gerber, P., *Lancet*, ii, 93–94 (1973).
7 Bernoco, D., *et al.*, *Folio Haemat.*, 54, 795–812 (1969).
8 Dick, H. M., Steel, C. M., and Crichton, W. B., *Tissue Antigens*, 2, 85–93 (1972).
9 Jeannet, M., and Alberto, P., *Schweiz. Med. Wschr.*, 102, 1170–1172 (1972).
10 Walford, R. L., Waters, H., Smith, G. S., and Sturgeon, P., *Tissue Antigens*, 3, 222–233 (1973).
11 Cullen, P. R., and Mason, D. Y., *Clin. exp. Immun.*, 17, 571–586 (1974).
12 Pious, D., Bodmer, J. G., and Bodmer, W. F., *Tissue Antigens*, 4, 247–256 (1974).
13 Bodmer, W. F., in *Histocompatibility Testing, 1972* (edit. by Dausset, J., and Colombani, J.), 611–617 (Munksgaard, Copenhagen, 1973).
14 Orkin, S. H., Buchanen, P. D., Yount, W. J., Reisner, H., and Littlefield, J. W., *Proc. natn. Acad. Sci. U.S.A.*, 70, 2401–2405 (1973).
15 Klein, E., *et al.*, *Cancer Res.*, 28, 1300–1310 (1968).
16 Goodfellow, P. N., *et al.*, *Nature*, 254, 267–269 (1975).
17 Allderdice, P. N., *et al.*, *Cell Sci.*, 12, 263–274 (1973).
18 Lamm, L. U., *et al.*, *Hum. Hered.*, 24, 273–277 (1974).
19 Bernoco, D., Cullen, S., Scudeller, G., Trinchieri, G., and Ceppellini, R., in *Histocompatibility Testing, 1972* (edit. by Dausset, J., and Colombani, J.), 527–537 (Munksgaard, Copenhagen, 1973).
20 McDevitt, H. O., and Bodmer, W. F., *Lancet*, ii, 1269–1275 (1974).
21 HL-A and disease, *Transplant. Rev.*, 22 (1975).
22 Jersild, C., Dupont, B., Fogh, T., Platz, P. J., and Svejgaard, A., *Transplant. Rev.*, 22, 148–163 (1975).
23 Winchester, R. J., *et al.*, *J. exp. Med.*, 141, 924–929 (1975).

Mouse T-cell surface glycoprotein recognised by heterologous anti-thymocyte sera and its relationship to Thy-1 antigen

CELL surface proteins specific for either T or B lymphocytes probably have a significant role in lymphocyte physiology, and we have therefore been characterising the plasma membrane proteins from mouse lymphocytes to identify such molecules and to determine their function. We have previously detected a high molecular weight T-cell-surface protein (T200, apparent molecular weight 200,000) which could be labelled by lactoperoxidase-catalysed iodination[1] and was recognised by rabbit anti-mouse thymocyte sera (ATS). We have now identified a low molecular weight T-cell-specific molecule present on mouse thymocytes, T lymphomas and peripheral T cells which is also recognised by rabbit antibodies to mouse lymphocytes.

This molecule was recognised during immunoprecipitation studies using labelled thymocytes and both unabsorbed ATS and a preparation of ATS made specific for T cells by absorption. ATS selectively precipitated two major iodinated species from labelled thymocytes—T200 and a low molecular weight component of broad electrophoretic mobility (T25, molecular weight 25,000–30,000; Fig. 1*b*). Several less heavily labelled species were also precipitated by ATS. Qualitatively similar results were obtained with four different preparations of antisera: two raised as described previously[2], one by repeated intravenous immunisation[3], and the fourth was a commercial preparation of antiserum (Microbiological Associates).

Similar gel profiles were observed whether cell lysates were prepared and immunoprecipitations carried out in the presence of either Nonidet P40 or sodium deoxycholate. Rabbit antisera to mouse T lymphomas that express the Thy-1 antigen also precipitate T25 and T200 (for example, anti-BW5147 serum, Fig. 1*e*, and anti-WEH1 22 serum, Fig. 1*f*). A preparation of ATS which was absorbed with neonatal liver cells and spleen cells from thymectomised, irradiated, neonatal liver cell-restored mice and was specific for mouse T lymphocytes as judged by indirect fluorescence and cytotoxicity studies (anti-T serum[4,5]), still contained antibodies which recognised T25 (Fig. 1*c*). This observation indicates that T25 carries antigenic determinants which are specific for T lymphocytes. The amount of radioactivity associated with T25 suggests that it is a major component of the thymocyte cell surface. Almost all the radioactivity precipitated by the anti-T cell serum was associated with T25 and this antiserum precipitated 4.8% of the total acid-insoluble radioactivity in an NP40 extract of labelled thymocytes. In contrast, unabsorbed ATS precipitated 8.5% and normal rabbit serum (NRS) 0.16% of the acid-insoluble radioactivity. T25 is also present on the surface of T lymphomas which express the Thy-1 antigen (for example, WEH1 22, Fig. 1*g*).

Examination of the labelled species precipitated by unabsorbed ATS or anti-T serum from iodinated peripheral lymphocytes of either normal or congenitally athymic (*nu/nu*) mice showed that T25 is present on peripheral T cells but probably in lesser amounts than on thymocytes (Fig. 2). Anti-T serum precipitated T25 from detergent-solubilised extracts of spleen and lymph node cells of normal mice (Fig. 2*c* and *e*) but not from peripheral lymphocytes of athymic mice. Unabsorbed ATS precipitated T25 from spleen and lymph node cells of normal mice, and T200 and the high molecular weight B-cell protein[1] from peripheral lymphocytes of both normal and athymic mice (Fig. 2*b*, *d* and *e*). A comparison of the gel profile of the labelled species precipitated by ATS with that of the labelled proteins precipitated by rabbit anti-mouse immunoglobulin serum (Fig. 2*h*) suggests that this particular preparation of ATS reacts with immunoglobulin.

Thus, T25 is a T-cell-specific molecule present on thymocytes, T lymphomas and peripheral T cells. Biosynthetic labelling studies, sensitivity to proteolytic enzymes and binding to plant
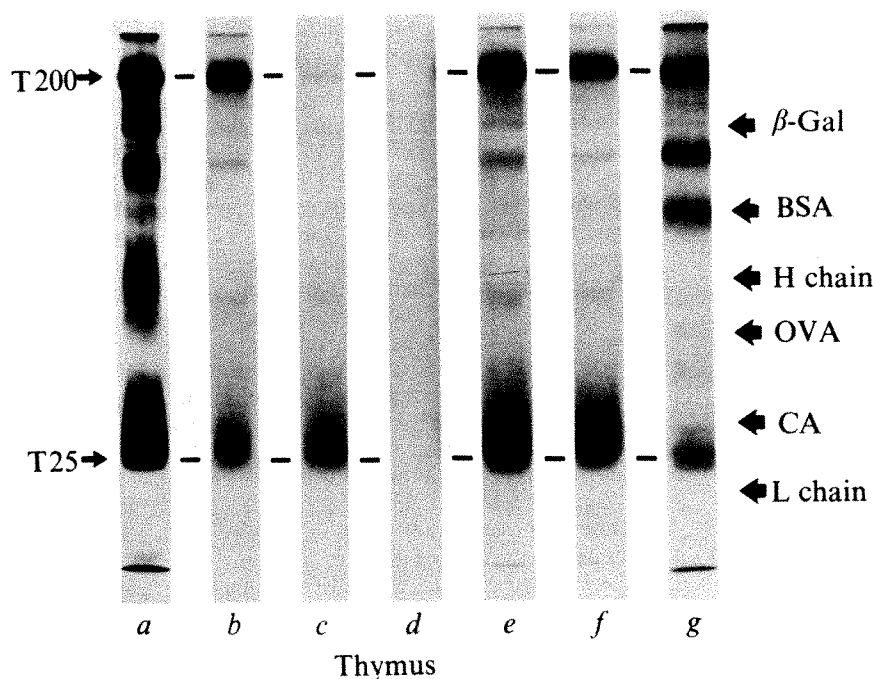
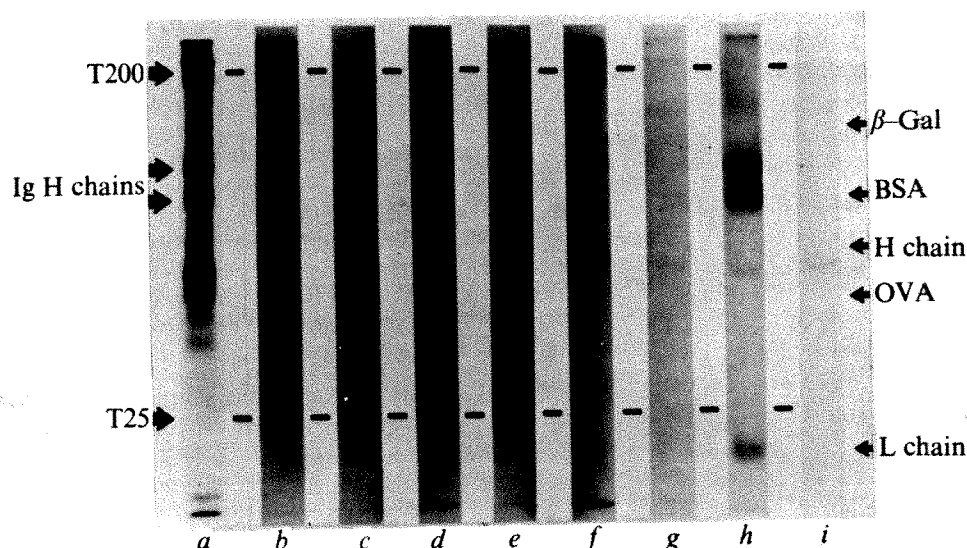Fig. 1 Autoradiographs of iodinated proteins of BALB/c thymocytes. *a*, Total thymocyte NP40 lysate. *b–f*, Immunoprecipitates of thymocyte NP40 lysate using various antisera: *b*, unabsorbed ATS; *c*, anti-T serum; *d*, NRS; *e*, anti-T lymphoma BW5147; *f*, anti-T lymphoma WEH1 22; *g*, immunoprecipitate of an NP40 cell lysate from WEH1 22 prepared with anti-WEH1 22 serum. Molecular weight standards: β-Gal, β-galactosidase; BSA, bovine serum albumin; H chain, rabbit IgG heavy chain; OVA, ovalbumin; CA, carbonic anhydrase; L chain, rabbit Ig light chain. A 9% polyacrylamide gel was used. The preparation of viable cell suspensions, lactoperoxidase-catalysed iodination, solubilisation of cells for immunological studies, and polyacrylamide gel electrophoresis in the presence of SDS have been described[1].

lectins suggest that T25 is a glycoprotein (I.S.T. and M.J.B., unpublished). Furthermore, no radioactivity was precipitated from an NP40 lysate of cells labelled with ^{3}H-palmitate using a variety of antisera which precipitate T25 and T200, suggesting neither molecule is associated with lipid in these conditions. Other investigations to be reported in detail elsewhere suggest that T25 is identical with the molecule which carries the mouse Thy-1 alloantigenic determinant[6]. T25 is precipitated by rabbit antisera against mouse brain, rat thymocytes and rat brain; similar cross reactivity has been noted for the Thy-1 molecule on the surface of mouse[7] or rat thymocytes[8]. T25 cannot be detected on the surface of Thy-1-negative variants[9] of mouse T lymphoma cell lines, and has the same properties as those of the rat thymocyte Thy-1 molecule[10] when chromatographed on Sepharose 6B in 1% sodium deoxycholate. T25 is recognised by a rabbit antiserum raised against a purified preparation of rat brain Thy-1. 1 antigen (Fig. 3), also indicating that T25 carries the Thy-1 determinant. This antiserum is specific for the Thy-1 molecule on rat thymocytes (A. N. Barclay, M. Letarte-Muirhead, and A. F. Williams, unpublished). Antibodies present in this antiserum which cross react with T25 from BALB/c thymocytes were removed by absorption with either mouse thymocytes, mouse brain homogenate, or rat thymocytes,

but not by rat lymph node cells which express only low levels of the Thy-1 antigen[11,12]. In contrast to this heterologous antiserum, mouse anti-Thy-1 alloantisera failed to specifically precipitate any iodinated species from thymocytes or T lymphoma cells whether solubilised in NP40 or sodium deoxycholate. This failure is probably a consequence of the low affinity of the anti-Thy-1 alloantibodies present in these sera. The presence of detergent or the abolition of multivalent interactions by disruption of the cell membrane may prevent formation of stable antibody–antigen complexes.

Two other possible explanations, that detergent specifically masks the Thy-1 antigenic determinant, or that the mouse Thy-1 antigenic determinant is a glycolipid[13,14] which is specifically associated with T25 on the T lymphocyte surface but which dissociates from T25 after solubilisation of the cell membrane by detergent can be excluded because an antiserum prepared from the rabbit anti-rat Thy-1 serum by absorption with BALB/c thymocytes or brain tissue precipitated T25 from an NP40 lysate of AKR/J thymocytes but not BALB/c thymocytes. The recognition of T25 by an antiserum apparently specific for the Thy-1. 1 alloantigenic determinant strongly suggests that T25 is identical with the Thy-1 antigen; only the unlikely possibility that BALB/c and AKR/J mice express

Fig. 2 Autoradiographs of iodinated peripheral lymphocytes from normal and congenitally athymic (*nu/nu*) mice: *a–c*, spleen cells; *d* and *e*, lymph node cells from BALB/c mice; *f–i*, mixture of spleen and lymph node cells of mice carrying *nu/nu* character backcrossed on to a BALB/c genetic background. *a*, Total spleen cell NP40 lysate; *b–i*, immunoprecipitates using various antisera; *b*, *d* and *f*, unabsorbed ATS; *c*, *e* and *g*, anti-T serum; *h*, anti-mouse Ig serum; *i*, NRS. A 9% polyacrylamide gel was used.
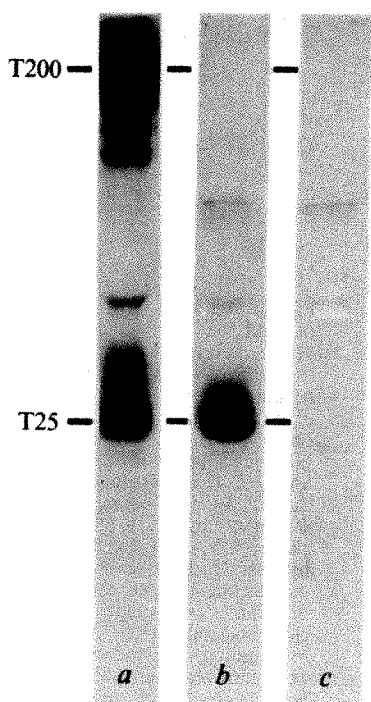
different alleles of another alloantigenic determinant with the same tissue distribution and interspecies cross reactivity as Thy-1, prevents these observations from being considered formal proof that T25 is the Thy-1 antigen. If this is the case then earlier reports that the mouse Thy-1 antigen is a glycolipid[13,14] require re-examination. One possibility is that the Thy-1 antigenic determinant may be carbohydrate; the blood group substances provide an example of a carbohydrate antigen present on both glycoprotein and glycolipid[15].

IAN S. TROWBRIDGE
IRVING L. WEISSMAN
MICHAEL J. BEVAN

The Salk Institute for Biological Studies,
PO Box 1809, San Diego, California 92112

1 Trowbridge, I. S., Ralph, P., and Bevan, M. J., *Proc. natn. Acad. Sci. U.S.A.*, 72, 157–161 (1975).
2 Levey, R. H., and Medawar, P. B., *Proc. natn. Acad. Sci. U.S.A.*, 56, 1130–1137 (1966).
3 Gutman, G., and Weissman, I. L., in *Morphological and Fundamental Aspects of Immunity* (edit. by Lindahl-Kiessling, K., Alm, G., and Hanna, M.), 595–602 (Plenum, New York, 1971).
4 Gutman, G. A., and Weissman, I. L., *Immunology*, 23, 465–479 (1972).
5 Friedberg, S. H., and Weissman, I. L., *J. Immun.*, 113, 1477–1492 (1974).
6 Reif, A. E., and Allen, J. M. V., *J. exp. Med.*, 120, 413–433 (1964).
7 Thiele, H.-G., and Stark, R., *Immunology*, 27, 807–813 (1974).
8 Morris, R. J., Letarte-Muirhead, M., and Williams, A. F., *Eur. J. Immun.*, 5, 282–285 (1975).
9 Hyman, R., and Stallings, V., *J. natn. Cancer Inst.*, 52, 429–436 (1974).
10 Letarte-Muirhead, M., Acton, R. T., and Williams, A. F., *Biochem. J.*, 143, 51–61 (1974).
11 Douglas, T. C., *Transplant. Proc.*, 5, 79–82 (1973).
12 Acton, R. T., Morris, R. J., and Williams, A. F., *Eur. J. Immun.*, 4, 598–602 (1974).
13 Vitetta, E. S., Boyse, E. A., and Uhr, J. W., *Eur. J. Immun.*, 3, 446–453 (1973).
14 Esselman, W. J., and Miller, H. C., *J. exp. Med.*, 139, 445–450 (1974).
15 Watkins, W. M., *Science*, 152, 172–181 (1966).

Thymus dependence of viral antigens

AFTER exposure of animals to most antigens, formation of antibody by cells of the B lymphocyte lineage requires helper effects of T lymphocytes[1]. The degree of thymus dependence varies for different antigens, and slowly metabolised immunogens with repeating epitopes, generally polysaccharides or polymerised forms of protein, seem to be the most thymus-independent[2]. Antigens of some viruses, however, may be relatively thymus independent. Inherent in the structures of many viruses is a rigid geometrical array of repeating antigenic determinants which, if the spatial relationships were appropriate, may effectively interact with and trigger B cells without help from T cells. Also, glycoproteins are important antigens in the virions of many viruses[3], and the carbohydrate portions of these molecules may function as thymus-independent immunogens. In addition, repeated immunisation of mice depleted of T cells can sometimes result in antibody production to thymus-dependent antigens[4], and virus infections often result in the sustained production of antigens and thus prolonged exposure of the infected host to the antigens. These antigens may be presented to the immune system in a variety of ways—on the membranes of infected cells, as soluble antigens released from such cells, and as repeating epitopes on virus particles.

The humoral antibody responses of athymic nude (*nu/nu*) mice after virus infection were compared with those of normal littermates (*nu/+* or *+/+*). Lymphocytes from nude mice have no demonstrable functions characteristic of T cells[5], whereas B cell functions such as blastogenic responses to lypopolysaccharide are present, and normal or increased antibody responses are made to thymus-dependent antigens such as type III pneumococcal polysaccharide[6]. The function of dendritic macrophages in nude mice is also normal[7] and nude mice can be rendered immunologically competent by the implantation of thymus grafts[8]. Heterozygous (*nu/+*) mice seem to be immunologically normal[5]. The alternative use of mice which have been depleted of T cells by thymectomy, irradiation, and bone-marrow reconstitution (TXBM) was rejected as such mice have defects in addition to T-cell depletion of T cells[9-11] (W. H. B. and H. C. Morse, unpublished).

Nude mice and their normal littermates were infected intraperitoneally and their sera assayed for antibody to certain antigens (Table 1). Sustained antibody responses to all 12 viruses from nine major virus groups were markedly thymus dependent. Nude mice, however, made early transient responses equal in magnitude to those of normal littermates to the picornaviruses, Coxsackie B1 virus and encephalomyocarditis (EMC) virus, to vesicular stomatitis virus (VSV), and to Sindbis virus. Antibody production in nude mice to the Sindbis antigen(s) that react with neutralising antibody was sustained for 2 weeks and was solely IgM as indicated by its appearance in the IgM fraction after column chromatography of serum on Sephadex G-200 and by its sensitivity to 2-mercaptoethanol (data not shown). Normal littermate mice produced IgG as well as IgM antibody to this antigen and these mice, unlike nude mice, produced large secondary responses (1:10,000) of predominantly IgG antibody. The Sindbis antigen(s) concerned are found on two virion membrane glycoproteins[12,13].

As Kennedy demonstrated that the antigenic determinants of Semliki Forest virus (an alphavirus similar to Sindbis virus) are in the polypeptide portions rather than the carbohydrate portions of the envelope glycoproteins[14], it will be of interest to determine if antibodies produced in the relatively thymus-independent IgM response react with antigenic determinants of Sindbis virus residing in the protein part of the molecule. Neutralising-antibody production by nude mice to Coxsackie B1 virus, EMC virus, and VSV peaked 6–8 d after infection, was sensitive to 2-mercaptoethanol, and thus was probably IgM immunoglobulin. As picornaviruses back lipid and carbohydrates, the neutralising antibody to Coxsackie B1

Table 1 Antibody titres in nude mice and littermate controls after virus infection

Genus	Virus	Assay*	Inoculum†	Immunisation schedule‡	Assay day§	Antibody titre Nude	Antibody titre Littermate
Picornavirus	Coxsackie B1	NT	10^5 PFU	0	6	512	512
			10^5 PFU	0	10	16	1,024
	Encephalomyocarditis	NT	10^3 PFU	0	7	256	256
			10^3 PFU	0	10	16	1,024
			10^3 PFU	0+10	17	<8	2,048
Orthomyxovirus	Influenza, PR8	HI	500 HAU	0	10	<2	256
			500 HAU	0	12	<2	256
			500 HAU	0	18	<2	256
			500 HAU	0	21	<2	128
Paramyxovirus	Simian virus 5	NT	10^3 PFU	0	7	<8	32
			10^3 PFU	0	10	<8	128
	Sendai	HI	100 HAU	0	13	<10	80
			100 HAU	0+14	21	<10	640
			100 HAU	0+14+21	28	<10	1,280
	Mumps	NT	10^4 PFU	0+13+26	33	<8	64
Rhabdovirus	Vesicular stomatitis	NT	10^6 PFU	0	7	256	512
			10^6 PFU	0	10	8	2,048
			10^6 PFU	0+10	17	16	>2,048
Togavirus	Sindbis, Egyptian 101	NT	10^5 PFU	0	2	32	32
			10^5 PFU	0	4	128	128
			10^5 PFU	0	6	256	256
			10^5 PFU	0	10	1,000	1,000
Parvovirus	Minute virus of mice	HI	10^5 PFU	0	7	<20	20
			10^5 PFU	0	9	<20	40
			10^5 PFU	0	10	<20	80
			10^5 PFU	0+12	17	40	160
			10^5 PFU	0+12+21	26	20	160
Adenovirus	Mouse adenovirus	CF	10^3 PFU	0	7	<10	<10
			10^3 PFU	0	11	<10	20
			10^3 PFU	0	15	<10	>80
Herpesvirus	Herpes simplex, type 1	NT	10^5 PFU	0	6	<8	64
			10^5 PFU	0	14	<8	128
Papovavirus	Simian virus 40	NT	10^7 PFU	0+5+17	27	<4	128

*NT, neutralising antibody; HI, haemagglutination-inhibiting antibody; CF, complement-fixing antibody.
†Immunising dose of infectious virus given intraperitoneally on day 0 and repeated thereafter as indicated in immunisation schedule.
‡Mice were inoculated on day 0 and on subsequent days as indicated.
§Antibody assays were carried out on indicated day after initial inoculation. Assays were carried out soon after infection to detect transient IgM responses, as well as late in infection and after multiple immunisations. Neutralisation tests were carried out by incubating 0.2 ml virus containing 100 plaque-forming units (PFU) and 0.2 ml diluted sera at 37 °C for 1 h and assaying for residual infectious virus. Complement-fixation (CF) and haemagglutination-inhibiting (HI) tests were carried out on coded serum samples by Microbiological Associates, Bethesda, Maryland. All sera were heated to 56 °C for 30 min before antibody assay. Titres are expressed as the reciprocal of the highest serum dilution that neutralised 50% of the virus and are the average of determinations on two or more animals.

virus and EMC virus must be directed against protein antigenic determinants. The neutralisation antigen(s) of VSV are found on an envelope glycoprotein, but the relative contributions of protein and carbohydrate to the antigenic determinants are not known.

Until availability of the athymic nude mouse, however, neonatal thymectomy or treatment with ALS was used to deplete animals of T lymphocytes and ascertain the thymus independence of viral antigens. Thus, neither neonatal thymectomy[4] nor ALS treatment[15] of mice diminished their production of HI antibody to influenza virus. In CF1 mice neonatally thymectomised, titres of complement-dependent neutralising antibody (probably IgM) to herpes simplex virus (HSV) were similar to those in control mice[16]. Discrepancies between these findings and the present demonstration of the thymus dependence of antibody production to influenza virus and HSV may stem from the use of mice inadequately depleted of T cells in earlier studies. Thymus dependence of HI antibody production to influenza virus has, however, been observed using TXBM[17] and nude[18] mice; the former do not produce neutralising antibody to HSV (W. H. B. and H. C. Morse, unpublished). Also, TXBM mice have been reported to respond well to Coxsackie B3 virus[19].

In conclusion, normal magnitude but transient IgM responses have been observed early after infection of athymic nude mice with several viruses. The antigens concerned are clearly protein in nature (picornaviruses) or are probably protein portions of virion envelope glycoproteins (Sindbis virus and VSV). The switch from IgM to IgG synthesis and the elicitation of secondary (IgG) antibody responses to these viral proteins, as to protein antigens generally, is highly thymus-dependent.

The present study has, however, surveyed the thymus dependence of a small number of viral antigens and utilised mice exclusively; the examination of other antigens of these same viruses, of different viruses, or the use of other animal species may disclose more thymus-independent viral antigens. Also, because of a limited supply of nude mice, dose–response studies were not carried out. Although normal littermates ($nu/+$ or $+/+$) were used in this study for comparisons, a more proper control would be thymus-grafted nude mice using thymuses from congenic mice. As the nude mice available were the result of passing the nu trait in outbred Swiss mice, such experiments were impossible and must await the availability of nude mice congenic with an inbred strain. The use of infectious agents makes difficult the administration of precise amounts of antigens, and the possibility exists that the viral infection itself may be thymus-dependent. Antigenic doses, however, were in some cases sufficient to elicit normal IgM responses in nude mice and the failure to switch to sustained IgG responses is unlikely to be caused by insufficient antigen.

Our findings should be considered in the light of the need for cell-mediated immunity in viral infections. Depletion of T cells may result in deficient antibody production as well as a diminution of effector cells involved in cell-mediated immunity. K-cell activity may also be diminished in those situations in which IgG antibody is not produced[20].

WILLIAM H. BURNS
LLOYD C. BILLUPS
ABNER LOUIS NOTKINS

National Institute of Dental Health,
National Institutes of Health,
Laboratory of Oral Medicine,
Bethesda, Maryland 20014

1 Miller, J. F. A. P., Basten, A., Sprent, J., and Cheers, C., Cell. Immun., 2, 469–495 (1971).

2 Sela, M., Mozes, E., and Shearer, G. M., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 2696–2700 (1972).
3 Burns, W. H., and Allison, A. C., in *Antigens*, 3 (edit. by Sela, M.), 479–574 (Academic, New York, 1975).
4 Svet-Moldavsky, G. T., Zinbar, S. N., and Spector, N. M., *Nature*, **202**, 353–355 (1964).
5 Wortis, H., *Contemp. Topics Immunobiol.*, **3**, 243–263 (1974).
6 Baker, P. J., Stashak, P. W., Amsbaugh, D. F., and Prescott, B., *J. Immun.*, **112** 2020–2027 (1974).
7 Mitchell, J., Pye, J., Holmes, M. C., and Nossal, G., *Austr. J. exp. Biol. med. Sci.*, **50**, 637–650 (1972).
8 Kindred, B., and Loor, F., *J. exp. Med.*, **139**, 1215–1227 (1974).
9 Howard, J. G., Christie, G. H., Courtenay, B. M., Leuchers, E., and Davies, A. J., *Cell. Immun.*, **2**, 614–626 (1971).
10 Feldmann, M., Wagner, H., Basten, A., and Holmes, M., *Austr. J. exp. Biol. med. Sci.*, **50**, 651–660 (1972).
11 Mitchell, G., and Humphrey, J., in *Germinal Centers of Lymphoid Tissue* (edit. by Jankovic, B., and Isakovic, K.) 125–131 (Plenum, New York, 1973).
12 Faulkner, P., and Dobos, P., *Can. J. Microbiol.*, **14**, 45–51 (1968).
13 Bose, H. R., and Sagik, B. P., *J. Virol.*, **5**, 410–412 (1970).
14 Kennedy, S. I. T., *J. gen. Virol.*, **23**, 129–143 (1974).
15 Hirsch, M. S., and Murphy, F. A., *Lancet*, ii, 37–40 (1968).
16 Mori, R., Kimoto, K., and Takeya, K., *Archs Virusforsch.*, **29**, 32, 38 (1970).
17 Virelizier, J. L., Postlethwaite, R., Schild, G. C., and Allison, A. C., *J. exp. Med.*, **140**, 1559–1570 (1974).
18 Haller, O., and Lindenmann, J., *Nature*, **250**, 679–680 (1974).
19 Woodruff, J. F., and Woodruff, J. J., *J. Immun.*, **113**, 1726–1734 (1974).
20 Cerottini, J., and Brunner, K., *Adv. Immun.*, **18**, 67–132 (1974).

Binding of α-foetoprotein to murine T cells

α-FOETOPROTEIN (AFP), an embryo-specific glycoprotein, is primarily known as a tumour-associated embryonic antigen. Although much is known about the occurrence of AFP in ontogeny and levels of AFP in different diseases, such as hepatocellular carcinoma and other tumours[1-4], little work has been done on its functional significance. Results from our laboratory have shown that AFP exerts an immunosuppressive effect on antibody synthesis when administered *in vivo*[5] and that it is a non-cytotoxic suppressor of both the primary and secondary antibody response to sheep red blood cells (SRBC) *in vitro*[6]. In addition, we found that AFP suppresses certain T-cell-dependent functions in mice such as allogeneic and mitogen-induced lymphocyte transformation[7]. A recent report[8] suggests that bovine foetuin is immunosuppressive for mitogen and allogeneic reactions. Bovine foetuin and AFP have, however, been shown to be different proteins[9]. Moreover, in view of the relatively large amounts of foetuin used to obtain suppression (10,000-fold higher than those required for suppression in our studies using AFP), it is possible that the foetuin preparations were contaminated with suppressive amounts of bovine AFP.

To define the cell type(s) affected by AFP we studied the binding of AFP to the surface of various cells involved in the immune reaction using direct immunofluorescence on preparations of thymus derived lymphochtes (T cells), bone marrow derived lymphocytes (B cells) and macrophages.

Mouse amniotic fluid (MAF) contains three major components: AFP, albumin, and transferrin[6]. MAF was collected from Ha/ICR mice in the late second trimester of pregnancy. Antiserum to MAF was prepared in rabbits by subcutaneous injection of MAF in Freund's complete adjuvant. A globulin fraction of this antiserum (double 18% sodium sulphate precipitation) was conjugated with fluorescein and absorbed with lyophilised normal adult mouse serum, rendering it monospecific for AFP. This antiserum gave a single line on Ouchterlony gel diffusion with MAF, which fused in a reaction of immunological identity with foetal, newborn and pregnant sera and purified AFP. Purified AFP was isolated from MAF as described previously[6]. Briefly, this involves affinity chromatography using an anti-whole adult mouse serum, followed by preparative polyacrylamide gel electrophoresis. The specificity of the AFP was determined by gel diffusion and immunoelectrophoresis using anti-mouse whole serum and anti-mouse MAF. The antisera were shown to be specific by the following: First, the absorbed antiserum gave a single line with whole concentrated MAF. This line fused on Ouchterlony with a single line in cord serum, pregnant serum and a highly purified preparation of AFP. Second, the antiserum was checked against another antiserum from Roswell Park, which is specific for AFP derived from a mouse hepatoma, and was found to form a line of

identity. Third, the fluorescent reaction was blocked by pure AFP and not albumin or transferrin (see ref. 6 for techniques of isolation and purity of preparations and antisera). In addition to the procedures outlined in that reference, we used gel filtration on Sephadex G-200. Finally, at least three other antisera have been produced in this laboratory recently against purified preparations of AFP and have been shown to be identical to the one used in the present study. No significant surface staining occurred with an anti-mouse albumin. (In addition the purity of the AFP was checked by sedimentation ultracentrifugation and polyacrylamide gel electrophoresis in both alkaline and sodium dodecyl sulphate gels.)

All studies were carried out using 6–10-week-old C57BL female mice with the exception of the experiment done with nude mice. We found that after a 90-min incubation in MAF a maximum number of cells stained in whole spleen preparation (approximately 18%) as shown by positive membrane fluorescence when reacted with anti-AFP. Various primary and secondary lymphoid tissues were examined to determine the presence of cells which bound AFP. The results presented in Table 1 show that spleen, lymph node and cortisone resistant thymocytes, but not bone marrow or nude mouse spleen cells, contained AFP binding cells.

Adult mouse spleen cells were treated with either anti-Fab or anti-θ plus complement to produce lymphoid preparations depleted of B and T cells respectively[10]. Spleen cells were passed sequentially over glass and nylon wool columns to enrich for T cells[11]. These preparations contained 88% cells staining with anti-θ and 7% cells staining with anti-Fab (anti-L chain). Peritoneal exudate cells were obtained by injection of thioglycolate to obtain a cell population containing a high proportion of macrophages[12]. Finally, a population of spleen cells was depleted of B cells and macrophages using glass and nylon wool columns and the non-adherent cells (largely T cells) were treated with anti-θ and complement. A summary of the results obtained for AFP binding on these various cell populations is presented in Table 2. AFP binds to cells which are not adherent to nylon wool and are destroyed by anti-θ plus complement. No binding is seen with B-cell enriched populations (spleen treated with anti-θ plus complement) or in the spleens of nude mice. We cannot rule out a small amount of binding to macrophages but the major cell type involved seems to be a T cell. Figure 1 illustrates the surface binding of AFP by spleen cells.

Since only a fraction (about one-third) of the T cells bind AFP, it is possible that a subclass of T cells may be involved in AFP suppression. More sensitive techniques, such as the use of ^{125}I-AFP, will, however, be necessary to determine whether significant numbers of molecules of AFP bind to "negative" T and B cells but are not present in sufficient numbers to be detected by the fluorescence method.

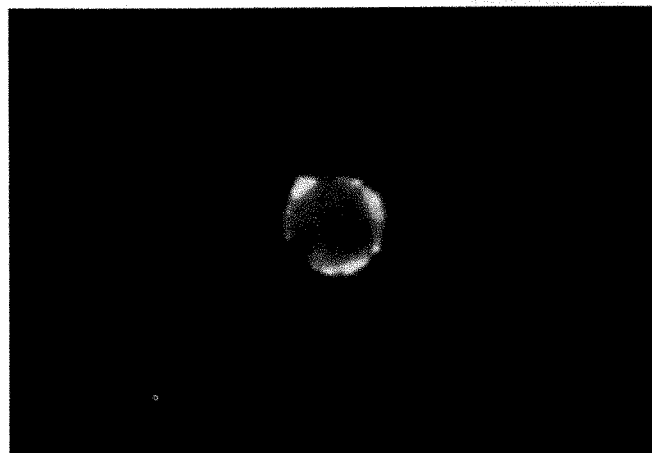The relatively small numbers of thymocytes ($< 1\%$) and

Fig. 1 A photomicrograph of C57BL mouse spleen cells incubated with MAF for 90 min, then stained with anti-AFP conjugated with fluorescein isothiocyanate.

Table 1 Percentage surface staining for AFP in the primary and secondary lymphoid tissues*

Treatment	Spleen	Lymph node	Thymus	Bone marrow	Cortisone resistant† thymocytes	Nude mouse] spleen cells
Media	< 1	< 1	<1	<1	<1	<1
NMS	< 1	< 1	<1	<1	<1	<1
MAF	18	23	<1	2	6	<1

*15×10^6 viable cells incubated in media, normal mouse serum (NMS) at 2 mg ml^{-1} or mouse amniotic fluid (MAF) at 2 mg ml^{-1}, for 90 min at 37 °C. Cells washed three times in cold media (RPMI 1640), suspended in 100 µl of media plus 50 µl of a fluoresceinated monospecific anti-AFP antiserum and incubated at 4 °C for 30 min. Surface fluorescence examined using a Leitz ortholux microscope. Figures are the average of duplicate experiments.

†Cortisone acetate (125 mg kg^{-1}) was injected intraperitoneally 2 d before the thymocytes were collected.

cortisone resistant thymus cells (6%) demonstrating binding compared with 18% of peripheral spleen T cells, suggest the possibility that AFP binding is detecting surface receptors which require additional maturation after peripheralisation from the thymus.

The binding of AFP to a T-cell population is compatible with the previous data from our laboratory showing that the addition of AFP late in the antibody response does not suppress the synthetic capacity of B cells[6]. We also reported[5,6] that the IgA and IgG responses are more sensitive to the suppressive effects of AFP than are IgM responses. These findings are consistent with AFP exerting its effect on helper T cells since the switch from IgM to IgG and IgA is thought to be T-cell dependent[13,14]. The observations[7] that AFP has a direct effect on certain T cell dependent functions (mitogen and allogeneic stimulation) in mice is consistent with the findings presented here. Finally, preliminary experiments (R.J.D., R. H. Keller, and T.B.T., unpublished) indicate that concanavalin A (con A) and methyl-α-D-glucopyranoside inhibit AFP binding suggesting that the con A and AFP binding sites may be identical or closely spaced on the membrane surface.

Table 2 Percentage surface staining for AFP*

Treatment	Spleen cells	Spleen cells passed over nylon wool†	Peritoneal exudate cells
Media	< 1	< 1	<1
NMS‡	< 1	< 1	<1
MAF	18	34	4
Anti-θ+complement then MAF	2	4	ND
Anti-Fab+complement then MAF	38	ND	ND

*Figures represent the averge of two separate experiments.
†Indirect immunofluorescence with anti-θ and anti-Fab showed that these preparations contained 88% θ-positive and 7% immunoglobulin-positive cells whereas the starting spleen cells contained 45% T and 44% B cells.
‡Normal mouse serum (NMS).
ND, Not done.

The mechanism of AFP suppression is unknown. T cells could be "turned off" by AFP binding to their surface, for example by mechanisms involving changes in cellular concentrations of cyclic AMP and cyclic GMP. Alternatively, AFP may activate suppressor cells or promote differentiation of a precursor T cell into a suppressor cell population. Further studies are necessary to define the mechanism(s) involved in AFP induced suppression.

RAYMOND J. DATTWYLER
ROBERT A. MURGITA
THOMAS B. TOMASI, JR

Department of Immunology,
Mayo Medical School,
Rochester, Minnesota 55901

1 Abelev, G. I., Transplant. Rev., 20, 3–37 (1974).
2 Abelev, G. I., Adv. Cancer Res., 14, 295–358 (1971).
3 Kew, M., Gut, 15, 814–821 (1974).
4 McIntire, K. R., Waldmann, T. A., Moertel, C. G., and Go, V. L. W., Cancer Res. (in the press).
5 Ogra, S. S., Murgita, R., and Tomasi, T. B., Immun. Commun., 3, 497–508 (1974).
6 Murgita, R., and Tomasi, T. B., J. exp. Med., 141, 269–286 (1975).
7 Murgita, R., and Tomasi, T. B., J exp. Med, 141, 440–452 (1975).
8 Yachnin, S., J. exp. Med., 141, 242–256 (1975).
9 Kithier, K., and Poulik, M. D., Biochim. biophys. Acta, 278, 505–516 (1972).
10 Stobo, J. D., Rosenthal, A. S., and Paul, W. E., J. Immun., 108, 1–17 (1972).
11 Julius, M. H., Simpson, E., and Heryenberg, L. A., Eur. J. Immun., 3, 645–649, (1973).
12 Ryder, R. J. W., and Schwartz, R., J. Immun., 103, 970–978 (1969).
13 Cough, J. D., Mims, L. H., and Strober, W., J. Immun., 106, 1624–1629 (1971).
14 Arnason, B. G., DeVoux St Cyr, C., and Relyveld, E.H., Int. Archs Allergy appl. Immun., 25, 206–224 (1964).

Hormonal regulation of α₁ foetoprotein

THE clinical usefulness of α_1 foetoprotein (AFP) is extensively documented[1] but the biological significance of this protein and the mechanisms regulating its synthesis remain unknown. We have investigated the effect of various hormonal, nutritional and haematological conditions on AFP metabolism in newborn and adult rats and mice. We present our results and some conclusions on regulation and function of AFP.

Different types of hormones markedly suppressed serum AFP levels in newborn rats. Among them, glucocorticoids (prednisolone 3 µg g^{-1} d^{-1}, dexamethazone 2 µg g^{-1} d^{-1}, hydrocortisone 15 µg g^{-1} d^{-1}) all induced a dramatic decrease in blood AFP (Fig. 1) together with an increase in albumin and total protein levels; this effect was partly reversible on cessation of treatment. Adrenocorticotrophic hormone (ACTH) (10 U d^{-1}), levothyroxine (3 µg d^{-1}), adrenaline (1:10,000, 2 µl g^{-1} d^{-1}) and db-cyclic AMP (10 µg g^{-1} d^{-1}) also produced significant decreases in AFP levels without depleting albumin and total protein levels. Inconsistent or non-significant effects were observed with desoxycorticosterone (0.025 µg g^{-1}d^{-1}), insulin (semilente 0.03 U g^{-1}d^{-1}), glucagon (0.1 µg g^{-1} d^{-1}), 17-β-oestradiol (3 µg g^{-1} twice a week), testosterone (3 µg g^{-1} twice a week) and progesterone (5 µg g^{-1} three times a week). Neonatal adrenalectomy, ovariectomy, orchidectomy, radiothyroidectomy or thymectomy did not modify the normal physiological disappearance curve of AFP. Adrenalectomy suppressed the effect of ACTH and reduced the effect of thyroxine but did not modify the effects of prednisolone and db-cyclic AMP. Thyroidectomy did not modify the effects of prednisolone, db-cyclic AMP or thyroxine.

Metabolic studies were carried out on prednisolone-, thyroxine- and adrenaline-treated rats. These three hormones did not significantly alter the serum clearance of radiolabelled AFP (half life 23 h), although they all decreased incorporation of ^{14}C-leucine in to AFP at a rate proportional to serum levels (Fig. 2). Liver tissue AFP content was also drastically decreased by prednisolone. These results indicate that prednisolone and probably adrenaline and thyroxine depress AFP levels through a selective blockade of its synthesis.

Attempts to correlate AFP levels with liver DNA synthesis activity of rats during uninterrupted prednisolone treatment disclosed the following facts. Prednisolone injection is rapidly followed by a marked depression of ^{3}H-thymidine incorporation in liver parenchymal cells. This negative phase, however, is always followed by a burst of labelling activity 8 d after injections are started (Fig. 1). The newly labelled cells are typical hepatocytes uniformly dispersed throughout the lobule.
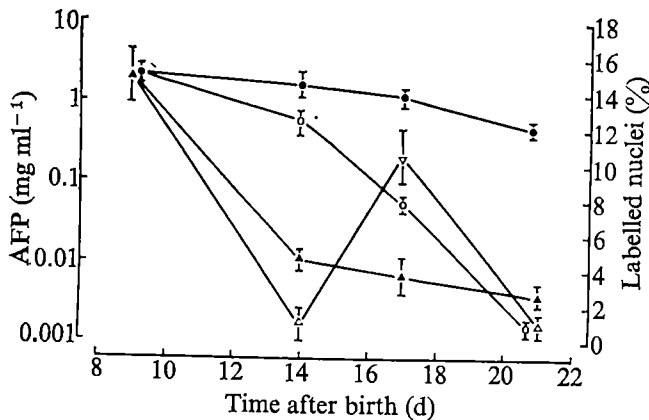
Fig. 1 Effect of prednisolone (3 μg g^{-1} d^{-1} from days 9 to 21) on serum AFP levels (●, ○) and ^{3}H-thymidine incorporation in liver cells of newborn rats (▲, △). Mean ±s.d. (4–6 rats). ●, ▲, Controls; ○, △, prednisolone. AFP-suppressive action of prednisolone was effective throughout the postnatal period (0–21 d) and also before birth (injection into amniotic sac of 16-d pregnant rats). The experiments were carried out on Sprague–Dawley rats and C3H/HeB mice. Hormones injected were current pharmaceutical products; usually, intraperitoneal injections were used, control animals receiving equivalent amounts of carrier. In neonatal experiments, half of every litter was used as control. Albumin and α$_1$ foetoprotein were quantitated by electroimmunodiffusion. The hepatic DNA synthesis activity was measured by autoradiography of liver tissue taken 1 h after intraperitoneal injection of ^{3}H-thymidine (2 μCi g^{-1}). Protein synthesis activity was evaluated by measuring the amount of radioactivity incorporated in serum AFP and total proteins 15–240 min after intravenous or intraperitoneal injection of ^{14}C-leucine (1.5 μCi per 100 g). The AFP catabolic rate was determined by measuring the serum clearance of ^{125}I-labelled antigen (purified by the method of Nishi[2]) between 6 and 168 h after injection of radioactive material (1 μCi per animal.)

This wave of DNA synthesis is never accompanied or followed by a resurgence of AFP secretion. It is in turn followed by another phase of suppression of ^{3}H-thymidine incorporation. Although the blocking action of glucocorticoids on liver DNA synthesis of newborn rats is a well known phenomenon[3], to our knowledge, such a biphasic effect of prednisolone has not been reported so far.

Our data thus indicate that adrenal and thyroid hormones may have an important function in the physiological regulation of AFP metabolism. Further, assuming that AFP is produced by a liver parenchymal cell and considering the association of AFP synthesis with cell division[4], the following conclusions may be reached. The secondary hepatocyte proliferation wave which appears during prednisolone treatment (Fig. 1) infers

Fig. 2 Effect of prednisolone (3 μg g^{-1} d^{-1} for 3 d) on ^{14}C-leucine incorporation in total serum proteins (*a*) and serum AFP (*b*) of 8-d-old rats. Radioactivity recovered from 5% TCA precipitates and from AFP–anti-AFP precipitates. Mean and limit values for 4–6 animals.
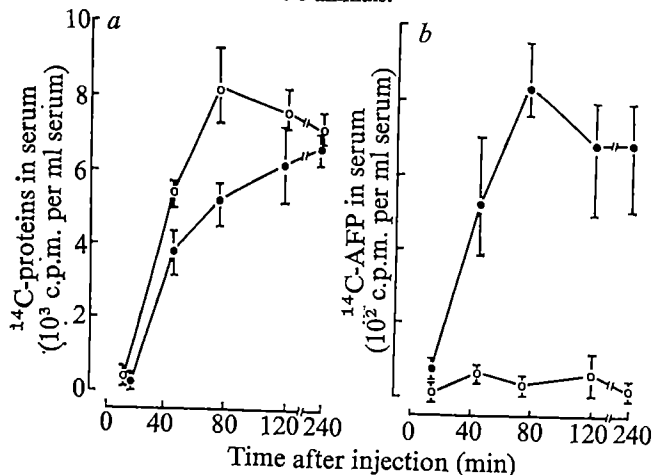
that AFP synthesis is not a prerequisite for hepatocyte proliferation; moreover, it shows that AFP is not produced indifferently by any type of mitotic hepatocyte. The rapid labelling of circulating AFP after ^{14}C-leucine administration (Fig. 2) indicates that AFP is rapidly secreted into the blood following its synthesis. Therefore, that AFP is synthesised before mitosis and released after mitosis[4–5] may be excluded; furthermore, injecting 4-d-old rats with colchicine (25 ng g^{-1}d^{-1} for 6 d) did not modify serum AFP levels (although it lowered by 47% the number of ^{3}H-thymidine-labelled liver cells).

In contrast to newborn rats (and mice), glucocorticoids usually have an enhancing effect on AFP levels in adult animals submitted to AFP-inducing conditions. This is best illustrated using mice intoxicated with CCl$_4$ (Fig. 3). In such animals, prednisolone markedly reduces the regenerative hepatocyte proliferation, but strikingly increases serum AFP levels; furthermore, the peaks of cellular proliferation and AFP secretion are shifted respectively from 40 to 72 h and from 3–4 to 6 d in control compared with hormone-treated animals (Fig. 3). Although prednisolone aggravates liver damage (as evidenced by levels of transaminases) and enhances passive release of AFP from injured cells, such opposite effects on AFP levels and cell proliferation clearly do not support the view that any dividing hepatocyte can resume AFP synthesis.

One interpretation would be that AFP synthesis can occur only during a definite stage of liver parenchymal cell ontogeny—in other words that AFP is the exclusive product of a 'transitional' cell, mitotically active and lying ontogenically between some stem cell (the 'oval' cell[6]) and the fully mature hepatocyte. This view is in very close agreement with the
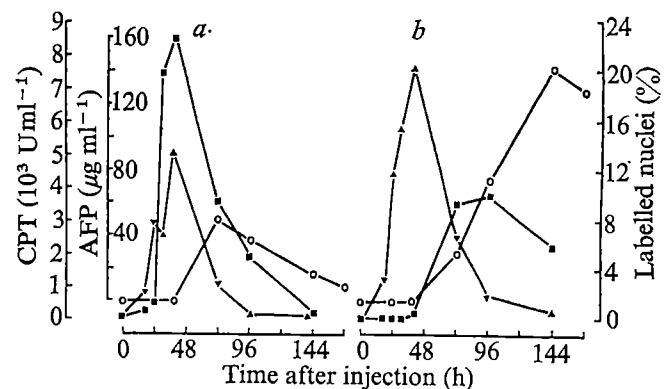
Fig. 3 C3H male adult mice injected intraperitoneally at 0 h with 0.1 ml CCl$_4$ per 100 g and from day 0 with daily subcutaneous injections of prednisolone (0.5 mg per animal) or saline (*a*). Each point is the mean of 4–6 animals. ▲, Cellular proliferation time (CPT); ○ AFP; radioactivity.

findings and hypotheses of Onoe *et al.* (see also Abelev[7]), and is supported by the work of Guegen *et al.* These authors have found that in newborn rat liver cell cultures, glucocorticoids promote the proliferation of mature hepatocytes but block (reversibly) the proliferation of 'intermediary cells'. If one assumes that such an intermediate cell is the AFP producer, this *in vitro* situation would account for the kinetics of AFP observed in newborn rats treated with prednisolone.

This could explain the lag phase between the hepatocyte division wave and peak of AFP production during liver regeneration. Being dependent on concomitant recruitment of progenitor cells, AFP production would take place during a later phase of liver repair, that is, during a fugaceaous intermediate mitotic stage of the stem cell on its way towards the mature hepatocyte. Thus, the effect of prednisolone on AFP levels during CCl$_4$ intoxication would be the result of a balance between strong indirect stimulation of stem cell recruitment due to enhanced liver damage and to reduced regenerative capacity of the hepatocytes and, as in the newborn, direct repression of the AFP-producing cell. At over 1 mg per animal

per d, the AFP-enhancing effect of prednisolone progressively decreases in CCl_4-treated mice, which would fit this scheme.

We also found that prednisolone stimulates AFP production in tumour (3'-Me-DABu-induced)-bearing rats. AFP production of rat hepatoma is related to tumour growth rate[9] and this enhancing effect of prednisolone is probably part of the representative response of tumours to glucocorticoids which seems to reflect the growth of the tissue[10].

We conclude that physiological regulation of AFP is influenced by all factors, such as hormones, which control and influence the growth or the maturation of the liver and, more specifically, the proliferation and the differentiation of the intermediate pre-hepatocyte cell. Therefore, all conditions which modulate or interfere with general physiological maturation processes could also affect AFP metabolism. One such hindering situation is neonatal iron deficiency, the effect of which on serum AFP levels is depicted in Fig. 4.
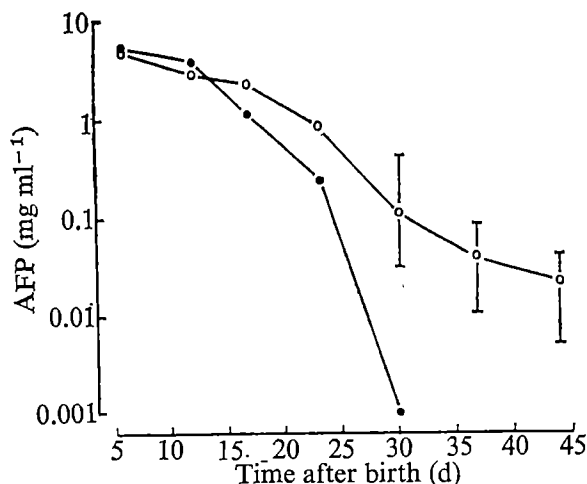

Fig. 4 Effect of iron deficiency on AFP levels of newborn rats. Deficiency was induced by serially bleeding gestating rats and by submitting them to an appropriate diet; weaned rats were maintained on the same diet. ●, Controls; O, iron-deficient. Each point is the mean of 4–7 animals taken from 2–6 litters. Where indicated, bars represent limit values.

There are obvious analogies between albumin and AFP. The two proteins share similar physicochemical properties, such as molecular weight, isoelectric point and electrophoretic mobility, and both have dye-binding properties[11] and hormone carrier functions[12]. The biological significance of AFP could be that of a foetal 'emergency' albumin, produced by a transitional parenchymatous cell committed to liver function but restrained in mitotic activity.

L. BELANGER*
D. HAMEL
L. LACHANCE
D. DUFOUR

Département de Biochimie,
Université Laval, Québec, Canada

M. TREMBLAY
P. M. GAGNON

Centre Hospitalier Universitaire,
Université Laval, Québec, Canada

1 Abelev, G. I., *Adv. Cancer Res.*, 14, 295–385 (1971).
2 Nishi, S , *Cancer Res.*, 30, 2507–2513 (1970).
3 Henderson, I. C., Fischel, R. E., and Loeb, J. H., *Endocrinology*, 88, 1471–1476 (1971).
4 Leffert, H. L., and Sell, S., *J. Cell Biol.*, 61, 823–829 (1974).
5 Sell, S., Nichols, M., Becker, F. F , and Leffert, H. L , *Cancer Res.*, 34, 865–871 (1974).
6 Onoé, T., Dempo, K., Kaneko, A., and Watabe, H., *Gann*, 14, 249–268 (1973).
7 Abelev, G. I., *Transplant. Rev.*, 20, 3–37 (1974).
8 Guegen, C., Guillouzo, A., Lenoir, P., and Bourel, M., *Biomedecine*, 21, 286–292 (1974).
9 Sell, S., and Morris, H. P., *Cancer Res.*, 34, 2489–2494 (1974).
10 Criss, W. E., and Morris, H. P., *Cancer Res.*, 33, 1023–1027 (1973).

11 Endo, Y., Kanai, K., Iino, S., and Oda, T., *Alpha-fetoprotein*, 47–54 (Colloque de l'INSERM, 1974).
12 Nunez, E., Engelmann, F., Benassayag, C., and Jayle, M. F., *C.r. hebd. Séanc. Acad. Sci., Paris*, 273, 831–834 (1971).

Functional evidence for postsynaptic supersensitivity of central noradrenergic receptors after denervation

THE development of postsynaptic or postjunctional supersensitivity in peripheral, noradrenergically innervated tissue after deafferentation has been clearly demonstrated[1-3]. In addition there exists considerable functional and biochemical evidence that central dopaminergic receptors in the neostriatum become supersensitive after lesions of the nigro-neostriatal projection[4-8]. Neurochemical evidence has been provided which is consistent with the development of postjunctional supersensitivity of central noradrenoceptors after deafferentation. Thus after intraventricular injections of 6-hydroxydopamine (6-OHDA), a neurotoxin for catecholaminergic neurones, noradrenaline (NA) stimulated synthesis of cyclic AMP is potentiated[9-11]. Functional evidence for the development of this type of supersensitivity in central NA receptors is, however, lacking. An enhanced response to intracisternally administered α-methylnoradrenaline in 6-OHDA pretreated rats has been reported[12] but such an effect could be mediated by changes in either pre- or postsynaptic mechanisms. Our experiments were designed to provide functional confirmation for the presence of postjunctional supersensitivity in central noradrenoceptors after lesions of central noradrenergic neurones. Clonidine [2-(2,6-dichlorophenylamino)-2-imidazoline] is a directly acting α-NA -receptor agonist[13-15] and has been shown to lower body temperature by a central α-noradrenergic mechanism. Thus intraperitoneal, intracisternal or intrahypothalamic injections of clonidine decrease body temperature[16,17]. It was hypothesised that if central noradrenoceptive neurones become supersensitive after destruction of noradrenergic afferents by 6-OHDA, then clonidine-induced hypothermia should be potentiated in lesioned animals.

Rats which received stereotaxic 6-OHDA injections which lesioned both dorsal and ventral noradrenergic projections (Fig. 1) did not differ significantly from controls on baseline (predrug) colonic temperature (control group 37.05 ± 0.17 °C; experimental group 37.3 ± 0.21 °C), although there was a tendency for the lesioned group to have slightly raised colonic temperature. The mean temperature of the lesioned group was lower than controls after each dose of clonidine, but this failed to reach statistical significance. Highly significant differences were, however, obtained when the temperature decreases (baseline minus postdrug temperature) were compared in the two groups. The hypothermic responses to several doses of clonidine in control and lesioned animals are given in Fig. 1. At each dose, clonidine produced a significantly greater drop in colonic temperature in the lesioned animals than in controls ($P < 0.01$). At the lowest dose administered (0.05 mg kg^{-1}), clonidine did not significantly reduce colonic temperature in the control group but did produce significant hypothermia in the lesioned animals ($P < 0.01$). Ten days after the last clonidine injection the animals were killed by cervical fracture and the brains were dissected into hypothalamus, hippocampus and cerebral cortex. Cortex and hippocampus were combined and NA was measured in each sample[19]. Control NA values were: hypothalamus 2.07 ± 0.03 μg g^{-1} and hippocampus–cortex 0.24 ± 0.01 μg g^{-1}. The 6-OHDA lesions reduced hypothalamic NA to 26% and combined hippocampal–cortical NA levels to 16% of control levels. These data indicate that the 6-OHDA produced extensive damage to both the dorsal and the ventral noradrenergic bundles[20]. Although not measured in our present experiments, identical lesions do not significantly affect striatal dopamine levels[21,22].

Because these resulted in significant damage to two of the

major ascending noradrenergic bundles, an additional experiment was done in an attempt to determine whether the increased hypothermic response to clonidine was a consequence of damage to the ventral or the dorsal NA projection. The surgical procedure was the same as above except that one group of animals ($n = 8$) received 6-OHDA lesions (4 μg per 2 μl) of ventral noradrenergic bundle ($A, -1.4$; $L, \pm 0.9$; and $DV, +1.0$ mm; coordinates taken from stereotaxic zero), whereas others ($n = 8$) received lesions to the dorsal noradrenergic bundle ($A, +2.6$; $L, \pm 1.1$; and $DV, +3.7$ mm; coordinates taken from stereotaxic zero). The injection rate was 0.2 μl min⁻¹. Control animals ($n = 8$) were sham operated. One month after the surgery, the animals were injected with clonidine-HCl (0.1 mg kg⁻¹) and colonic temperature was measured 120 min later. The baseline (preclonidine) temperatures of the three groups did not differ significantly although there was a tendency for the ventral bundle lesioned animals to be slightly hyperthermic (controls 37.04 ± 0.17 °C; dorsal noradrenergic bundle 37.0 ± 0.11 °C; ventral noradrenergic bundle 37.48 ± 0.10 °C). Clonidine significantly decreased rectal temperature in each group ($P < 0.01$). Compared with controls, however, only the animals receiving ventral noradrenergic bundle lesions showed a significant enhancement of the hypothermic response to clonidine ($P < 0.01$). The temperature decrease was 0.67 ± 0.17 °C in controls, 0.75 ± 0.28 °C in the dorsal bundle lesioned group and 1.30 ± 0.11 °C in ventral bundle lesioned rats. The biochemical results of this experiment are given in Table 1. Ventral noradrenergic bundle lesions reduced hypothalamic NA by 89%, but did not have a statistically significant effect on hippocampal–cortical levels of NA. Lesions aimed at the dorsal noradrenergic bundle on the other hand reduced levels of hippocampal–cortical NA by 94% and hypothalamic NA by 69%.

Our results indicate that central noradrenoceptors, like central
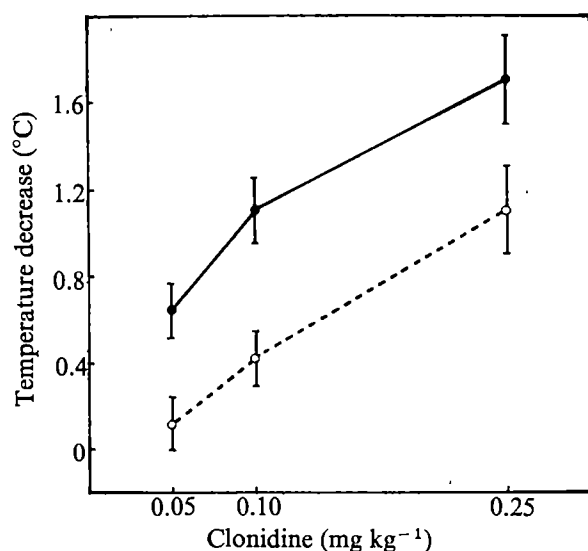
Fig. 1 Effect of clonidine-HCl on body temperature in controls (○) and in rats receiving 6-OHDA lesions of ascending noradrenergic projections (●). The 6-OHDA injections were aimed at the ascending noradrenergic projections caudal to the substantia nigra in male Wistar rats (280–320 g). The coordinates were: $A, +1.0$ mm; $L, \pm 1.3$ mm; and $DV, -2.1$ mm according to König and Klippel[18]. 6-OHDA hydrobromide was injected bilaterally (4 μg in 2 μl, dosage expressed as the base, dissolved in 0.15 M saline containing 0.2 mg ml⁻¹ ascorbic acid) through a 34 gauge needle at a rate of 0.4 μl min⁻¹. Control animals underwent sham operations. Three months after the surgery, both groups received intraperitoneal injections of clonidine-HCl in the order 0.1, 0.05 and 0.25 mg kg⁻¹ (dosage expressed as the salt) with 4 d between each drug administration. Colonic temperature was measured before and 120 min after the injection and was determined by inserting a probe 4 cm into the rectum. Ambient temperature was 22–24 °C Data represent means ±s.e.m. of 8 animals in each group. The groups differed significantly at each dose ($P < 0.01$).

Table 1 Effect of 6-OHDA lesions of the dorsal or ventral noradrenergic bundles of hypothalamic and hippocampal–cortical levels of NA

	Hypothalamic NA (μg g⁻¹)	Hippocampal–cortical NA (μg g⁻¹)
Controls	2.17 ± 0.08	0.24 ± 0.01
Dorsal noradrenegic bundle lesion	0.66 ± 0.06* (31%)	0.014 ± 0.01* (6%)
Ventral noradrenergic bundle lesion	0.24 ± 0.04* (11%)	0.21 ± 0.01 (85%)

Data represent means ±s.e.m. of 8 animals in each group. Numbers in parentheses indicate percentage of control values.
*Significantly different from controls ($P < 0.01$).

dopamine receptors, undergo changes after deafferentation which are consistent with the postjunctional supersensitivity hypothesis. Thus, the dose–response curve of the hypothermic response to clonidine, a noradrenoceptor agonist, was shifted to the left in animals bearing lesions of at least two of the major ascending noradrenergic projections (Fig. 1). Such a shift in the dose–response curve has been taken as a primary indicator of postjunctional supersensitivity[1-3]. Reid[23] found that the hypothermic effect of clonidine (0.5 mg kg⁻¹) was not significantly increased 7–12 d after intracisternal injections of 6-OHDA, although there was a tendency for the experimental animals to show a greater drop in temperature. The reason for this discrepancy is not clear but may result from differences in procedure, or it may be that Reid did not obtain sufficient reductions in NA. Another criterion for postjunctional supersensitivity is that the enhanced response has a gradual onset and increases with time. Further studies will be required to determine if the enhanced hypothermic effect of clonidine satisfies this second criterion.

In the second experiment, an attempt was made to determine to what extent lesions of the dorsal or ventral noradrenergic bundles contributed to the observed effects. Although the lesions were not completely effective in destroying one system without damaging the other, reasonably discrete lesions were obtained (Table 1). It is obvious from the results of this experiment that the potentiation of clonidine-induced hypothermia was a consequence of damage to the ventral noradrenergic bundle. These data suggest, therefore, that clonidine produces hypothermia by an action on central noradrenoceptors which are normally innervated by the ventral noradrenergic bundle. They also suggest that this ascending system, which terminates mainly in the hypothalamus[20], may have an important role in thermoregulation. It would be of interest to determine to what extent animals with lesions of the ventral noradrenergic bundle may have thermoregulatory deficiencies.

A. P. ZIS
H. C. FIBIGER

Division of Neurological Sciences,
Department of Psychiatry,
University of British Columbia,
Vancouver, Canada V6T 1W5

1 Trendelenburg, U , *Pharmac. Rev.*, 15, 225–276 (1963).
2 Trendelenburg, U., *Pharmac. Rev*, 18, 629–640 (1966).
3 Fleming, W W., McPhillips, J. J., and Westfall, D P , *Ergebn. Physiol.*, 68, 55–119 (1973).
4 Ungerstedt, U , *Acta physiol. scand., Suppl.*, 367, 69–73 (1971).
5 Von Voigtlander, P. F , and Moore, K. E., *Neuropharmacology*, 12, 451–462 (1973).
6 Price, M. T. C., and Fibiger, H. C., *Eur. J. Pharmac.*, 29, 249–252 (1974).
7 Mishra, R. K , Gardner, E. L , Katzman, R., and Makman, M. H., *Proc. natn. Acad. Sci. U.S.A.*, 71, 3883–3887 (1974).
8 Fibiger, H C , and Grewaal, D S., *Life Sci.*, 15, 57–63 (1974).
9 Palmer, G. C., *Neuropharmacology*, 11, 145–149 (1972).
10 Kalisker, A , Rutledge, C. O., and Perkins, J. P., *Molec Pharmac.*, 9, 619–629 (1973).
11 Huang, M., Ho, A. K. S., and Daly, J W., *Molec. Pharmac.*, 9, 711–717 (1973).

12 Breese, G. R., Moore, R A , and Howard, J. L., *J Pharmac. exp. Thér* , 180, 591–602 (1972)
13 Andén, N.-E., *et al.*, *Life Sci.*, 9, 513–523 (1970).
14 Corrodi, H., Fuxe, K., Ljungdahl, Å., and Ogren, S. O., *Brain Res* , 24, 451–470 (1970).
15 Haeusler, G , *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, 286, 97–111 (1974).
16 Laverty, R., and Taylor, K M., *Br J. Pharmac* , 35, 253–264 (1969).
17 Tsoucaris-Kupfer, D., and Schmitt, H , *Neuropharmacology*, 11, 625–635 (1972).
18 Konig, J. F. R., and Klippel, R. A , *The Rat Brain* (Williams and Wilkins, Baltimore, Maryland, 1963).
19 McGeer, E G , and McGeer, P L , *Can. J. Biochem. Physiol* , 40, 1141–1151 (1962).
20 Ungerstedt, U., *Acta physiol. scand., Suppl* , 367, 1–48 (1971)
21 Fibiger, H. C , Phillips, A G., and Zis, A. P., *Pharmac. Biochem. Behav.*, 2, 87–96 (1974)
22 Roberts, D C S , Zis, A. P., and Fibiger, H C., *Brain Res.* (in the press).
23 Reid, J L , in *Advances in Neurology*, 9 (edit by Calne, D. B , Chase, T N , and Barbeau, A), 73–80 (Raven, New York, 1975)

# Spontaneous voltage fluctuations in retinal cones and bipolar cells

TRIFONOV[1] and others have suggested that vertebrate rods and cones release transmitter continuously in the dark and that the effect of light is to suppress this release by making the inside of the cell more negative. On this hypothesis the bipolar cells, which receive information from cones, should be electrically noisy in the dark, because of random fluctuations in the release of cone transmitter, and relatively quiet in the light when the release of transmitter is suppressed[2].

Such an effect is illustrated in Fig. 1a, which is an intracellular recording from a hyperpolarising bipolar cell in the isolated eyecup of the turtle, *Pseudemys scripta elegans*. In darkness, the cell voltage fluctuated at low frequency in an apparently random manner over a range of about 5 mV peak-to-peak. Steady illumination of the centre of the receptive field produced a maintained hyperpolarisation of about 6.5 mV and a considerable reduction in noise. When the light was turned off, there was a brief depolarising transient of almost 30 mV followed by slow recovery of both dark voltage level and noise. The voltage variance was 1.53 mV^2 in darkness and 0.045 mV^2 in light.

The possible order of magnitude of the events underlying the dark noise of a bipolar cell may be estimated as the change in voltage variance divided by the change in mean voltage[7] and is found to be 0.24 mV. This is similar to the amplitude of a miniature end-plate potential (e.p.p.), but the ionic movement underlying the event must be much smaller than that in a miniature e.p.p. since the input resistance of bipolar cells is likely to be two or three orders of magnitude higher than that of a muscle fibre. At present it is uncertain whether the hypothetical events underlying the bipolar cell noise are analogous to the effects produced by single acetylcholine molecules or to those of the packets of molecules which produce miniature e.p.ps.

There was often no significant and reproducible change in variance when the bipolar cell depolarised in response to an annulus of light, but in some cells a consistent reduction in noise did occur. This is illustrated in Fig. 1b, where the dark variance of 0.072 mV^2 was reduced to 0.029 mV^2. Since depolarisation of bipolar cells by an annulus is mediated by hyperpolarisation of luminosity horizontal cells[3,6], these findings suggest that the horizontal cells liberate hyperpolarising transmitter at a relatively high rate in darkness and at a lower rate when hyperpolarised.

Figure 2 illustrates the unexpected result that a steady light suppresses noise in cones as well as in bipolar cells. The record in Fig. 2a was obtained on a red-sensitive cone, which from its small receptive field was taken to be an isolated cone not coupled to its neighbours[8]. The cell was unusually noisy and the dark variance of 0.40 mV^2 was reduced to 0.025 mV^2 during illumination which hyperpolarised by about 14 mV at the initial peak. Effects of this kind were observed consistently, although the cells were usually less noisy than in the example given. A more typical result was obtained from a green-sensitive cone (Fig. 2b) in which light reduced the dark variance of 0.018 mV^2 to
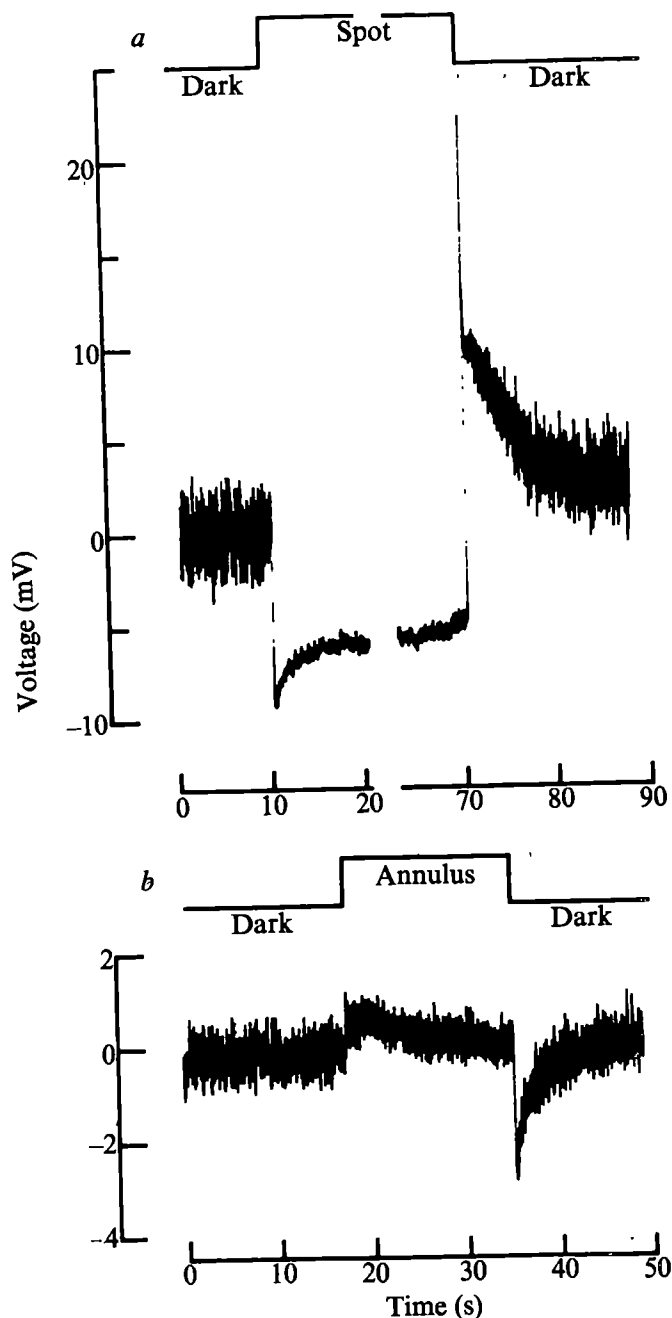
Fig. 1 Intracellular recordings from centre-hyperpolarising, red-sensitive bipolar cells Abscissae measure time and ordinates give membrane potential relative to mean voltage in darkness (called zero). a, Response to a 210-μm diameter centred spot which delivered 4 13 × 10^4 photon μm^{-2} s^{-1} to the retina at 639 nm; b, Response of another cell to an annulus of 630 μm internal diameter and 1,300 μm external diameter; photon flux was the same as in (a). The relatively low variance and small light response are attributed to damage by the impaling microelectrode. A PDP-11 computer (DEC) was used to digitise the signals and calculate the variances; dark values given in the text are the mean of measurements taken before and after illumination. 1,024 points were sampled at 5 ms intervals after passing the signal through an RC high-pass filter with 1 s time constant and a 3-pole low-pass filter with 50 Hz cutoff frequency. Micropipettes were drawn from capillary tubing containing a fused partition ("θ tubing"), as they were found to fill consistently and to show sufficiently low intrinsic noise Bipolar cells and cones (Fig. 2) were identified by electrical criteria established from dye marking experiments[3-6].

0.004 mV^2 or less (less because much of the noise in the light may be instrumental in origin). Since the spatial properties of cells with low electrical noise indicate that they are coupled electrically to neighbouring cones, it seems likely that coupling
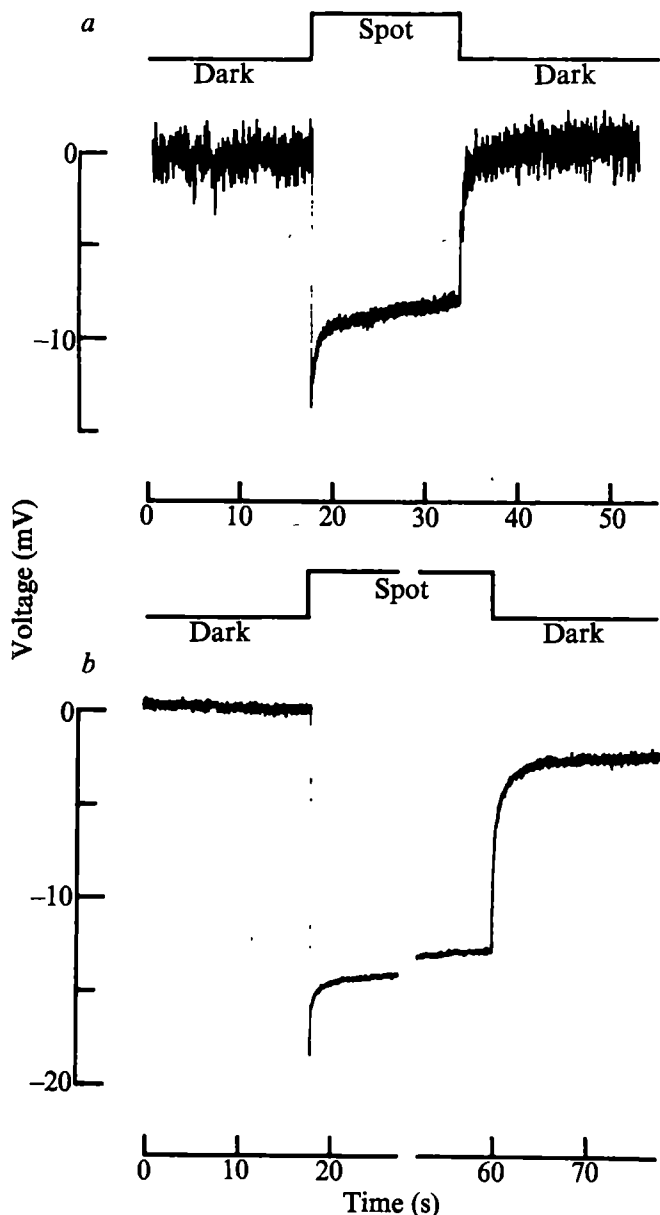
Fig. 2 Recordings from cones in darkness and during steady central illumination. *a*, Response of an isolated red-sensitive cone to a 6-μm spot delivering 8.05×10^5 photon μm^{-2} s^{-1} at 639 nm; *b*, Response of a green-sensitive cone to a 132-μm spot delivering 1.67×10^6 photon μm^{-2} s^{-1} at 559 nm. The relatively low noise in the dark is characteristic of coupled cones.

reduces noise (see refs 9, 10). Lights which hyperpolarised by more than a few mV consistently reduced noise, but very dim lights gave no significant change and it is uncertain whether the initial slope of the curve relating noise to light intensity is positive or negative.

It is surprising to see noise suppression in a cone, because the natural expectation is that random absorption of quanta should give 'photon noise' as it does in a photomultiplier tube or in an invertebrate photoreceptor[11]. The synaptic action of horizontal cells on cones[6] might contribute to the variance in the dark, but this cannot account for the main observation because small areas of illumination (Fig. 2) suppress the noise even though their effect on horizontal cells is very small. It therefore seems necessary to suppose that there is a source of electrical noise in the dark which is reduced as the cone is hyperpolarised by light and more than compensates for the fluctuations introduced by the random absorption of light quanta. A possible

source of "dark noise" would be random closure of the light-sensitive ionic channels either spontaneously or as a result of variations in the concentration of a blocking or opening substance. In the kinetic model of Baylor *et al.*[12], it is assumed that light releases a blocking molecule which hyperpolarises the cell by closing ionic channels. If the relationship between hyperpolarisation (*u*) and the concentration (*z*) of the blocking molecule is of the form

$$u/u_{max} = z/(z+K)$$

and if the variance of *z* is proportional to *z*, then the variance of *u* should be maximal when $z = K/3$ and $u = u_{max}/4$ (ref. 7). In terms of this hypothesis, our data require that the dark value of *z* be about $K/3$ and that the cell be hyperpolarised in darkness by about 5 mV from the potential it would have if all the light-sensitive ionic channels were open all of the time.

E. J. SIMON
T. D. LAMB
A. L. HODGKIN

Physiological Laboratory
University of Cambridge,
Cambridge CB2 3EG, UK

1 Trifonov, Y. A., *Biofizika* (in Russian), 13, 809-817 (1968)
2 Falk, G., and Fatt, P , in *Handbook of Sensory Physiology*, VII/1 (edit by Dartnall, H. J. A.), 200-244, (Springer, New York, 1972).
3 Werblin, F. S., and Dowling, J. E , *J Neurophysiol* , 32, 339-355 (1969)
4 Kaneko, A , *J Physiol* , Lond , 207, 623-633 (1970).
5 Baylor, D. A., Fuortes, M. G. F , and O'Bryan, P M , *J. Physiol* , Lond , 214, 265-294 (1971).
6 Richter, A., and Simon, E J , *J. Physiol* , Lond , 248, 317-334 (1975).
7 Katz, B., and Miledi, R., *J. Physiol., Lond.*, 224, 665-699 (1972).
8 Baylor, D A , and Hodgkin, A. L , *J. Physiol., Lond* , 234, 163-198 (1973)
9 Fain, G. L., *Science*, 187, 838-841 (1975).
10 Schwartz, E. A , *J. Physiol* , Lond., 246, 617-638 (1975).
11 Fuortes, M G. F., and Yeandle, S., *J gen Physiol* , 47, 443-463 (1964)
12 Baylor, D. A., Hodgkin, A. L., and Lamb, T D., *J. Physiol* , Lond , 242, 685-727 (1974).

Evidence that sensory axons are mitogenic for Schwann cells

WE have obtained preparations of peripheral nerve tissue in culture which provide either Schwann cell populations, essentially free of connective tissue cells such as fibroblasts, or outgrowing neurites from sensory (or autonomic) ganglia entirely free of ensheathing Schwann cells or connective tissue elements. Thus, these two major elements of peripheral nerve can be studied and characterised separately or in recombination. We report here that the 'bare' sensory ganglion neurite provides a potent stimulus for thymidine incorporation and mitosis in Schwann cells.

Tissue culture techniques utilised in obtaining separation of these two components from the rat peripheral nervous system will be reported in detail elsewhere. Briefly, to obtain Schwann cells, rat dorsal root ganglia (DRG) obtained just before birth were stripped of their capsule and after explantation were exposed to cytosine arabinoside (ara-C)[1] and fluorodeoxyuridine (FdU)[2]. Cultures were fed with medium containing these compounds at 10^{-5} M concentrations during days 1 and 3 *in vitro*, and with regular medium during day 2 and subsequently. After 10 d *in vitro* ganglia were transplanted to new culture dishes where they provided a fresh outgrowth of neurites and Schwann cells within 5–7 d, and this outgrowth was essentially free of connective tissue cells. The neurites could then be easily eliminated from these cultures by removing the ganglionic mass containing the neurone somata (Fig 1*a*). After neurite breakdown the population of Schwann cells previously related to the neurites was retained. This population

is hereafter referred to as a Schwann cell bed. To obtain unensheathed neurites, the schedule of exposure of DRG to ara-C and FdU was continued through day 7 *in vitro* in alternation with the regular medium as described above. In addition, after day 7 the cultures were maintained on medium containing FdU, which suppresses subsequent Schwann cell proliferation. After about 1 month of continued exposure to FdU, Schwann cells could not be detected in the outgrowth arising from the ganglion. The ganglion could then be transplanted to a new dish where it provided a fresh growth of neurites completely free of Schwann cells in the absence of antimitotic agents. These cultures are hereafter referred to as bare neurite preparations.

Schwann cell beds prepared as described above may contain fibroblasts in some cases. In the experiments to be described below the incidence of fibroblasts was so infrequent that they did not interfere with the observations. In addition, fibroblasts in culture are easily distinguished from Schwann cells on the basis of their larger size, flatter shape and lack of specific relationship to neurites[3]. We have observed that with two additional 1-d exposures to ara-C and FdU it is possible to completely eliminate fibroblasts from the cultures, and to obtain populations of Schwann cells virtually free of fibroblasts. Our general culture technique uses a collagen substrate in a modified Petri dish, a medium containing 25% human placental serum and 10% 9-d chick embryo extract (50% in saline), nerve growth factor, and a 5% CO_2–95% air atmosphere[4].

Autoradiography after application of ^{3}H-thymidine (0.5 μCi ml^{-1} in regular medium) to Schwann cell beds on successive days after ganglion removal demonstrates a decreasing number of labelled Schwann cells over a period of about 10 d; after this time less than five cells per hundred are labelled. It is then possible to transfer ganglia providing bare neurites into these culture dishes, both in areas occupied by Schwann cells and in areas free of Schwann cells. In more than ten separate experiments of this type we have consistently observed that: the presence of a ganglion providing bare neurites located remote from the Schwann cell bed has no effect on the Schwann cell labelling index; Schwann cells in beds only partly invaded by neurites exhibit a stimulation of ^{3}H-thymidine incorporation

only in those bed regions directly invaded by bare neurites (Fig. 1, *a–c*); the degree of stimulation provided by bare neurites is dramatic, with about 90% of the Schwann cells in the invaded area incorporating ^{3}H-thymidine after exposure for 42 h. The number of Schwann cells greatly increases in areas invaded by neurites and frequent mitotic figures are seen. Ganglia providing bare neurites not placed near Schwann cell beds do not generate a Schwann cell population, indicating that the implanted ganglia did not obtain this capability during the course of the experiment.

One of these experiments in which a ganglion providing bare neurites was placed along the border of an extensive Schwann cell bed is shown in Fig. 1. The labelling index is greatly increased in the zone of interaction between neurites and Schwann cells, but not elsewhere. The neurite population on the opposite side of the implanted ganglion is, at this point, essentially cell-free, but in preparations kept for a week or more after this stage Schwann cell invasion of the entire neurite halo occurs. The identity of these cells as Schwann cells is based not only on their general size and morphology (Fig. 1*b* and *c*) but also on electron microscopic observations of their distinctive ensheathing relationship to the axon.

The literature on Schwann cell proliferation during development and nerve injury contains many instances where the proliferative capability of the Schwann cell population is documented (reviewed in ref. 5). It is clear that Schwann cell proliferation in damaged nerve has often been ascribed to the presence of degeneration products within the nerve; the growing axon has not generally been considered a factor in Schwann cell proliferation; and there has been little discussion of the possible mechanism by which Schwann cell numbers are controlled during embryonic development. Our observations indicate that the presence of a growing nerve fibre during either initial growth or regeneration must be considered a potent stimulus to Schwann cell proliferation; there may, of course, be other stimuli.

The demonstrated mitogenic capacities of the axon may explain several recent *in vivo* observations. Thomas[6] has observed that repeated crushes (which enable optimal axon
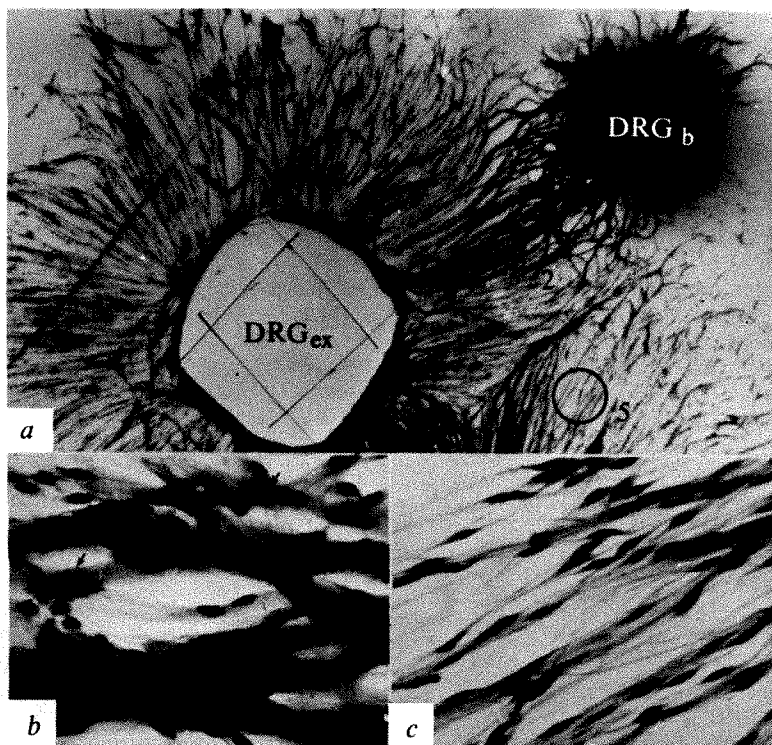
Fig. 1 *a*, Autoradiogram illustrating interaction between bare neurites and quiescent Schwann cells. Schwann cells derived from a dorsal root ganglion explant extend radially from the vacated site of the excised ganglionic mass (DRG$_{ex}$). A second ganglion (DRG$_b$) from which Schwann cells had been eliminated (by previous treatment with ara-C and FdU) was implanted in an area adjacent to the Schwann cell bed 8 d after excision of the first ganglion. Within 3 d bare neurites from the second ganglion (DRG$_b$) invaded the Schwann cell bed and the culture was exposed at that time to ^{3}H-thymidine for 42 h. The culture was then fixed with 4% formaldehyde in phosphate buffer, processed for whole mount autoradiography using Kodak NTB2 emulsion, and stained with erythrosin β-Toluidine blue stain. At this low magnification the Schwann cell nuclei appear as small specks with the Schwann cell processes aligned in a radial manner from the zone marked DRG$_{ex}$. Darkly stained processes originating at DRG$_b$ interact with and become unsheathed by Schwann cells derived from DRG$_{ex}$ but remain unensheathed, at this early stage, on the opposite side of the ganglion. Counts of labelled and unlabelled Schwann cell nuclei were made in the fields designated by the circles. Counts were: 1, 115 (labelled)/15 (unlabelled); 2, 31/71; 3, 0/120; 4, 0/129; 5, 1/59. ×18. *b*, Higher magnification of field 1 shown in *a*. This field clearly lies in the zone of interaction between Schwann cells and bare neurites. Most of the detectable Schwann cell nuclei in this area are covered with silver grains; only a few unlabelled nuclei (arrows) are present. ×204. *c*, Higher magnification of field 4 shown in *a*. In contrast to *b* this field has not been invaded by bare neurites from the implanted DRG and none of the nuclei is labelled. In general the labelling index in quiescent Schwann cell beds is less than 5%, compared with a labelling index of more than 80% in areas where proliferation is stimulated by actively growing neurites. ×204.

regrowth) of a peripheral nerve leads to a remarkable over-production of Schwann cells in the distal nerve stump reaching a point at which these cells are applied in multiple layers around each single axon. Also, in crush lesions of the abnormal peripheral nerve roots of dystrophic mice, where a paucity of Schwann cells and myelin segments provides much less degenerating material than in normal nerve, an increase in the number of myelin segments and, presumably, Schwann cells is induced by crush injury[7]. The importance of the axon in provoking Schwann cell proliferation is also indicated by observations, on the proximal stump of severed or crushed sciatic nerve, that proliferation occurs independently of widespread nerve fibre or myelin degeneration and in conditions in which nerve regeneration is most actively occurring[8].

Our observation of the axon–Schwann cell interaction resulting in mitogenesis illustrates one use of cultures containing either 'pure' Schwann cell populations or bare neurite preparations. These preparations may be useful in further analysis of the interaction between neurite and Schwann cell, as well as in studies of the basic properties of the Schwann cell itself.

This work was supported by the National Multiple Sclerosis Society.

PATRICK M. WOOD
RICHARD P. BUNGE

Department of Anatomy,
Washington University School of Medicine,
St Louis, Missouri 63110

1 Fischbach, G. D., *Devl Biol.*, **28**, 407 (1972).
2 Bunge, R., Rees, R., Wood, P., Burton, H., and Ko, C. -P., *Brain Res.*, **66**, 401 (1974).
3 Murray, M., and Stout, A. P., *Am. J. Path.*, **16**, 41 (1940).
4 Bunge, R. P., and Wood, P. M., *Brain Res.*, **57**, 261 (1973).
5 Causey, G., *The Cell of Schwann* (Livingston, Edinburgh and London, 1960).
6 Thomas, P. K., *J. Anat.*, **106**, 463 (1970).
7 Stirling, C. A., *Brain Res.*, **87**, 130 (1975).
8 Logan, J. E., Rossiter, R. J., and Barr, M. L., *J. Anat.*, **87**, 419 (1953).

Biological reactivity of PVC dust

CONCERN has been expressed about the biological potential of polyvinyl chloride (PVC) and its associated monomer vinyl chloride monomer (VCM)[1]. During the industrial processing of PVC, workers can be exposed to varying quantities of this material in the form of a dust. At the present time, however, there is little detailed biological or biochemical information on the effects of inhaled or ingested PVC, or on the reactivity of this dust material. The biological reactivity of other dusts (silica, asbestos) have been studied by a haemolysis technique[2] which is useful for assessing the degree of membrane-induced damage by a variety of toxic materials. Other *in vitro* screening systems using lung[3] and other cells[4] have also provided useful information on structural and biochemical changes induced by particulate matter. Here we report on the haemolytic potential of PVC dust, comparison of which is made with the highly haemolytic and biologically reactive chrysotile asbestos A (UICC standard reference sample) and the effect of PVC on lung fibroblast cultures.

The haemolysis technique used has been described in detail previously[2]. The degree of haemolysis was expressed as a percentage of the totally lysed sample and the results presented as the means and ranges (if any) of, at least, quadruplicate assays. Two samples of PVC were tested (KM 1 and KM2) both of which were obtained as finely-divided dried powders which had been formed by standard processing procedures for use in fabrication work.

The first sample (KM 1) was highly haemolytic at relatively low concentrations, 100% haemolysis (over 50 min) being achieved by between 7.5 and 10 mg of dust (Fig. 1*a*). With increasing concentrations of PVC, haemolysis seems to be reduced, but this effect is most likely the result of some of the released haemoglobin binding to the dust, which is then spun down into the pellet. Sample KM 2 PVC was found to be practically non-
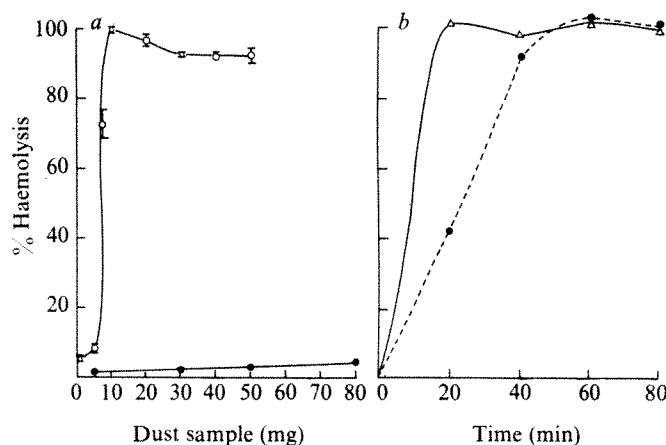
Fig. 1 Haemolysis by PVC dusts and chrysotile asbestos. *a*, Haemolytic potency of KM 1 (○) and KM 2 (●) PVC samples after 50 min; *b*, change in haemolysis with time by 7.5 mg samples of UICC chrysotile asbestos A (△) and KM 1 (●) PVC. Briefly, a 1 % (v/v) suspension of packed rabbit erythrocytes in veronal buffered saline, pH 7.4, was used in all the experiments. The incubation mixture normally consisted of dust sample with 1 ml 1 % erythrocyte suspension + 3 ml veronal buffered saline. After incubation and agitation for 50 min (or varying time intervals) at 37 °C the samples were centrifuged at 2,000 r.p.m. for 20 min and the extinction of the supernatant read at 541 nm. Controls consisting of a totally lysed sample (1 ml 1 % erythrocyte suspension + 3 ml water) and a fragility control (1 ml 1 % erythrocyte suspension + 3 ml veronal buffered saline) were treated identically in each experiment.

haemolytic. A sample of 100 mg of KM 2 PVC gave an equivalent haemolytic effect to 1 mg of sample KM 1. The haemolytic activity with time of a sample of KM 1 PVC (7.5 mg) was compared with an equivalent mass of chrysotile asbestos A (Fig. 1*b*). It is evident that the asbestos is a faster haemolytic agent, although total lysis is achieved by PVC after 1 h.

During the processing of different forms of PVC, a variety

Fig. 2 Haemolysis by washed samples of KM 1 PVC dust (7.5 mg) and the resulting supernatant fluids from these washings compared with the haemolytic activity of the untreated dust sample. KM 1 PVC *a*, Untreated; *b*, washed once; *c*, washed twice; *d*, washed three times; *e*, wash 1; *f*, wash 2; *g*, wash 3.
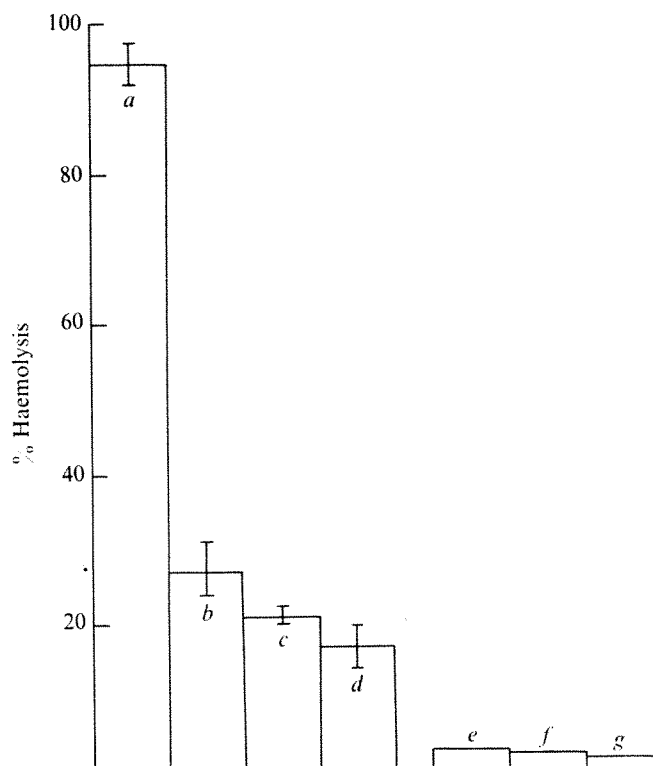
of agents are used which could contribute to the haemolytic effect of the dust. Therefore, samples (7.5 mg) of KM 1 PVC were washed once, twice and three times (5 s each wash, in 3 ml veronal buffer on a whirlimix; followed by centrifugation, 2,000 r.p.m. for 20 min) and the haemolytic potency of the washed dust samples and the supernatant PVC fluid were compared with the lytic potential of an untreated PVC sample (Fig. 2). After a single wash, the haemolytic potency of KM 1 PVC is reduced by over 60% and subsequent washes reduce the activity of the dust even further. Thus the removal of some surface-associated material, which must exist in a reasonably soluble form, considerably reduces the biological potency of this PVC dust. It is also evident that this material, once removed from the dust surface and diluted out in solution, has very limited haemolytic activity (Fig. 2).

The effect of KM 1 PVC was studied on the levels of cell mat DNA, RNA, protein and hydroxyproline (assessment of collagen) in lung fibroblast cultures maintained *in vitro* for 24 d. The methods of isolation and cell culture and the analyses for DNA, RNA protein and hydroxyproline have been detailed previously[3,5]. Before addition of different concentrations of KM 1 PVC (50–200 µg ml^{-1} culture medium or 0.5–2.0 mg per culture) the dust sample was first washed in a balanced salt solution containing antibiotics (see legend to Fig. 3). Other methods of dust sterilisation (heat, autoclaving, radiation)

Fig. 3 The effect of different concentrations of KM 1 PVC on lung fibroblast cultures. The PVC sample was first washed with an antibiotic solution (penicillin (100 units) streptomycin (100 µg) in 1 ml) and then added as a single dose to 3-d-old cultures (in logarithmic growth). Cultures were maintained in 10 ml of 20% foetal bovine serum plus Waymouth's medium containing additional ascorbic acid[5], changed twice weekly and removed for analysis on day 24.

were not considered to be feasible and while the washing procedure undoubtedly reduces the ability of this PVC sample to damage cell membranes (Fig. 2) fibroblast cultures treated with the dust all had lower levels of cell mat hydroxyproline after 24 d (Fig. 3). Some fluctuation in DNA levels was apparent with different dust concentrations but the significance of these is doubtful without further experimentation. There is little change in the level of total protein or RNA in the PVC-treated cultures.

From these preliminary findings we conclude that certain

forms of PVC dusts exhibit a high haemolytic potential because of the presence of a readily soluble, surface-associated agent. Exposure to this type of PVC dust may thus constitute an additional health hazard because of the increased biological activity of the dust. Work is in progress to determine the nature of the haemolytically-active agent by assessing a wide range of PVC dusts of which complete knowledge has been obtained of the chemical processing. The possibility that VCM is the active agent has been explored, but both samples tested were found to contain immeasurable amounts of VCM (<1 p.p.m.). Nevertheless, the present study indicates that the introduction of a washing procedure after the processing of KM 1 PVC dust would certainly reduce or abolish the haemolytic activity of this material.

R. J. RICHARDS
RASHMIBALA DESAI
P. M. HEXT
F. A. ROSE

*Department of Biochemistry,
University College, PO Box 78,
Cardiff CF1 1XL, UK*

[1] Selikoff, I. J , and Hammond, E. C , (Eds), *Toxicity of Vinyl chloride–Polyvinyl chloride, Ann. N Y. Acad. Sci.*, 246, 377 (1975)
[2] Harington, J. S , Miller, K., and MacNab, G., *Environ. Res* , 4, 95-117 (1971).
[3] Richards, R. J., and Morris, T. G , *Life Sci* , 12, 441-451 (1973).
[4] Allison, A., Harington, J. S., and Birbeck, M. J , *J exp. Med.*, 124, 141-161 (1966).
[5] Richards, R. J., and Wusteman, F. S , in *Tissue Culture in Medical Research*, 91-99 (Heinemann, London, 1974).

Mechanism of induction of haemolytic anaemia by phenylhydrazine

A COMPOUND with the optical spectrum of a ferrihaemochrome was produced when ferricyanide-oxidised phenylhydrazine was added to a solution of ferrihaemoglobin[1]. The three isomers of methylphenylhydrazine similarly resulted in ferrihaemochromes, but 4-hydrazinobenzoic acid did not[2]. The induction of haemolytic anaemia by a substituted phenylhydrazine was related to the reactivity of its oxidised form with ferrihaemoglobin to produce a ferrihaemochrome[2,3]. Further studies of the reaction of oxidised arylhydrazine with ferrihaemoglobin have established that the formation of a ferrihaemochrome-like product and the character of the optical spectrum of this product depend on the nature and position of substituents on the benzene ring of phenylhydrazine.

Substituted phenylhydrazine hydrochlorides were obtained from commercial sources or were synthesised by standard procedures. Each compound was purified by recrystallisation from 2 N HCl or ethanol, and the structure and purity of the recrystallised product were confirmed by its NMR spectrum, infrared spectrum, melting point, and analyses for C, H, N, and Cl. Oxyhaemoglobin solutions were deoxygenated by the passage of oxygen-free[4] nitrogen or helium, and the resulting ferrohaemoglobin was oxidised to ferrihaemoglobin by the addition of excess ferricyanide. To a solution of ferrihaemoglobin in excess ferricyanide, a solution of arylhydrazine hydrochloride in water or ethanol was added, and the optical spectrum that resulted was recorded with the Cary Model 17 instrument. The spectra obtained with 3- and 4-chlorophenylhydrazine were similar to that previously reported with unsubstituted phenylhydrazine[1]. The spectra obtained with 3,4- and 3,5-dichlorophenylhydrazine were distinguished by a more prominent absorption band in the red than the others. 2-Chloro- and 2,3-, 2,4-, and 2,5-dichlorophenylhydrazine resulted in spectra with a single broad maximum at around 540–550 nm and without a band in the red. 2-6-Dichlorophenylhydrazine, 2,6-dimethylphenylhydrazine, 2-hydrazinobenzoic

acid, and 4-hydrazinobenzoic acid each resulted in slow and incomplete formation of ferrihaemochrome. On the other hand, the ethyl ester of 4-hydrazinobenzoic acid resulted in the quantitative formation of a ferrihaemochrome. Results with the isomers of dichlorophenylhydrazine are shown in Fig. 1.

Arylhydrazine hydrochloride and potassium ferricyanide were mixed in oxygen-free solution, and the reduction of ferricyanide was followed at 420 nm (Fig. 2). In agreement with the stoichiometry of the oxidation of arylhydrazine to aryldiazene[5,6], 2 mol of ferricyanide were rapidly consumed per mol of phenylhydrazine or 4-hydrazinobenzoic acid; further reduction of ferricyanide was very slow. 2,6-Dimethylphenylhydrazine, not shown here, also reduced two equivalents of ferricyanide very rapidly. 2,6-Dichlorophenylhydrazine, on the other hand, reduced ferricyanide more slowly, but after 10 min had consumed more oxidant than either phenylhydrazine or 4-hydrazinobenzoic acid.

The finding that each substitution on the benzene ring of phenylhydrazine resulted in a ferrihaemochrome with a different optical spectrum indicates that the ferrihaemochromes are compounds of ferrihaemoglobin with ligands that include the substituted benzene ring of the oxidised arylhydrazines and supports the suggestion that the optical spectrum obtained when oxidised phenylhydrazine was added to ferrihaemoglobin resulted from the binding of phenyldiazene $(C_2H_5N = NH)^1$. The bond was postulated to form between the iron atom of ferrihaem and the nitrogen atom bound to the benzene ring in the phenyldiazene molecule[5]. The low reactivity of 2,6-dichlorophenyldiazene and of 2,6-dimethylphenyldiazene can be explained by the steric hindrance of their respective di-*ortho* substituents to the formation of such a bond. The reactivity of oxidised eth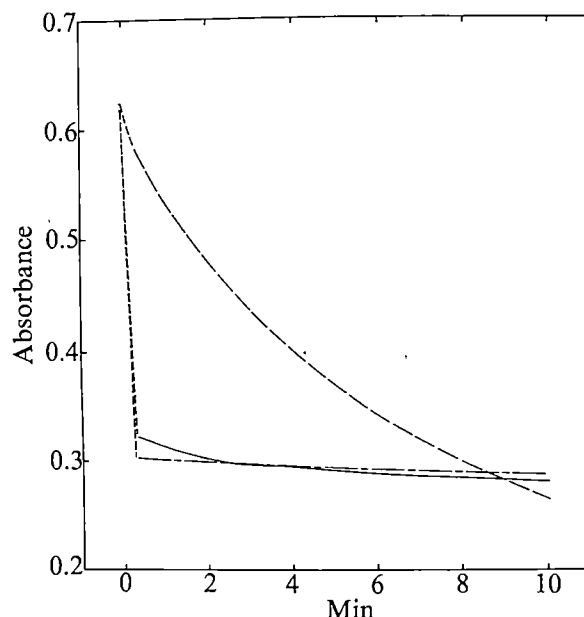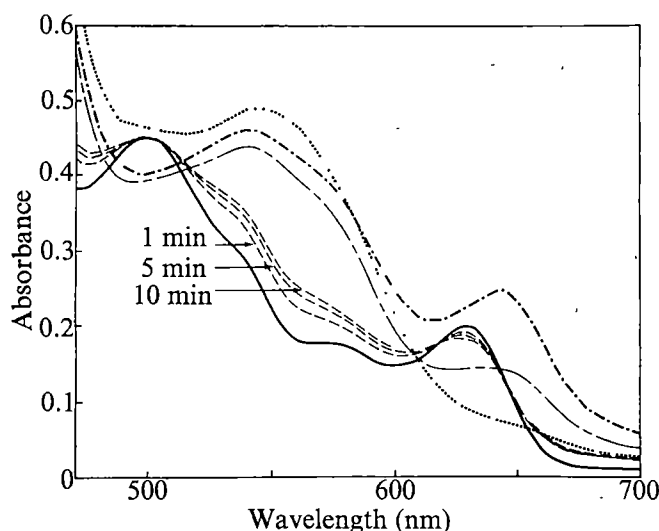yl 4-hydrazinobenzoate suggests that the low reactivity of oxidised 4-hydrazinobenzoic acid is due not to the ring position or size of the carboxyl group, but to its negative charge.

The effect of adding arylhydrazine to oxyhaemoglobin was examined with the compounds of Fig. 2. Phenylhydrazine

Fig. 2 Oxidation of arylhydrazines by potassium ferricyanide. The disappearance of the absorption peak of potassium ferricyanide at 420 nm was followed at 23 °C. Absorbance of 6.0× 10^{-4} M potassium ferricyanide (......). Interval between addition of arylhydrazine hydrochloride and beginning of absorbance record (--------). Absorbance after arylhydrazine hydrochloride was added to make 1.5×10^{-4} M solutions of: phenylhydrazine (————); 2,6-dichlorophenylhydrazine (—————); 4-hydrazinobenzoic acid (– – –).

caused a rapid change in colour to greenish brown followed by progressive precipitation of protein. 4-Hydrazinobenzoic acid resulted in complete replacement of oxyhaemoglobin by a mixture of ferrihaemoglobin (75%) and ferrihaemochrome (25%) without precipitation. 2,6-Dichlorophenylhydrazine resulted in slow transformation of oxyhaemoglobin to 15% ferrihaemoglobin after 2.5 h. Beaven and White[7] reported that 2- and 4-nitrophenylhydrazine reacted with oxyhaemoglobin to produce precipitates of "green haemoglobin" but that 2,4-dinitrophenylhydrazine produced only ferrihaemoglobin. We found that 2- and 4-nitrophenylhydrazine reacted with ferrihaemoglobin in the presence of excess ferricyanide to form ferrihaemochrome-like compounds but that 2,4-dinitrophenylhydrazine did not. Substituted phenylhydrazines, including ethyl 4-hydrazinobenzoate, that resulted in the quantitative formation of ferrihaemochromes also induced haemolytic anaemia in experimental animals while 2- and 4-hydrazinobenzoic acid, 2,6-dichlorophenylhydrazine, and 2,6-dimethylphenylhydrazine did not[2,8]. Thus, the ability of an arylhydrazine to cause both oxidative denaturation of oxyhaemoglobin and haemolytic anaemia in animals paralleled the ability of a product of its oxidation to react rapidly and quantitatively with ferrihaemoglobin to produce a ferrihaemochrome.

Phenylhydrazine results in the formation of ferrihaemoglobin from oxyhaemoglobin[9], and phenylhydrazine is oxidised to phenyldiazene by oxygen[5]. The two products, ferrihaemoglobin and phenyldiazene, react rapidly and quantitatively with each other or form a ferrihaemochrome. All three reactions are essential because 4-hydrazinobenzoic acid, which was shown to take part in the first two but not the third of these reactions, did not result in oxidative denaturation or anaemia. An amino acid replacement in the haem pocket destabilises haemoglobin by breaking non-polar contacts between haem and globin[10] and results in unstable haemoglobin haemolytic anaemia[11]. The binding of phenyldiazene was postulated to destabilise haemoglobin in phenylhydrazine-induced haemolytic anaemia[5]; however, the acuteness of the latter anaemia contrasts with the chronic nature of the former. The production of free radicals and hydrogen peroxide in the reduction of molecular oxygen and the bound oxygen of oxyhaemoglobin by phenyl-

Fig. 1 Spectra of ferrihaemoglobin (————) and of ferrihaemochromes produced by oxidised dichlorophenylhydrazines: 2,3-dichloro (- - - - - - - - -); 3,4-dichloro (- — - — - —); 3,5-dichloro (- ———— ——); 2,6-dichloro (— — — —). To 4.00 ml of 5.0×10^{-5} M ferrohaemoglobin in 0.05 M sodium phosphate (1:1) buffer (*p*H 6.84), 25 or 26 μl of 0.1 M potassium ferricyanide and 12 μl of 0.05 M arylhydrazine hydrochloride were added in succession. Spectra were recorded 1, 5, and 10 min after the addition of arylhydrazine. All three recordings are shown for 2,6-dichlorophenylhydrazine. Reactions with the other isomers were nearly complete after 1 min, and only the 5-min spectra are shown. The 10-min spectra showed little change. The spectra resulting from the 2,4-dichloro and 2,5-dichloro isomers were very similar but not identical to that resulting from the 2,3-dichloro isomer

hydrazine[12-14] can account for the marked difference in rate of haemolysis. Abnormally high concentrations of these reactive oxidants would lead to an accelerated rate of oxidation of the porphyrin rings[15,16] and the thiol groups[17] of phenylhydrazine-induced ferrihaemochrome. The resulting disruption of normal non-polar interactions between haem and globin and between the polypeptide chains of globin would then cause rapid formation of Heinz bodies, the presence of which is associated with haemolysis[18]. The induction of haemolytic anaemia only by those arylhydrazines that resulted in the quantitative formation of ferrihaemochromes favours a molecular mechanism for the induction of oxidative denaturation by an arylhydrazine in which the oxidation of susceptible groups within the haemoglobin molecule is facilitated by the destabilising effect of a large ligand that includes the aryl portion of the arylhydrazine.

This work was supported by research grants from the National Institutes of Health.

HARVEY A. ITANO
KAZUHIRO HIROTA
KEIMEI HOSOKAWA

Department of Pathology,
University of California, San Diego,
La Jolla, California 92037

[1] Itano, H. A, and Robinson, E. A., *J Am. chem. Soc.*, 83, 3339–3340 (1961).
[2] Itano, H A., Hollister, D. W, Fogarty, W. M. Jr, and Mannen, S, *Proc Soc. exp Biol. Med*, 147, 656–658 (1974).
[3] Itano, H. A., Hosokawa, K, Hirota, K., and Mannen, S, *Fedn Proc.*, 34, 654 (1975)
[4] Meites, L., and Meites, T., *Analyt. Chem.*, 20, 984–985 (1948)
[5] Itano, H A., *Proc. natn. Acad Sci. U.S.A.*, 67, 485–492 (1970).
[6] Mannen, S, and Itano, H A., *Tetrahedron*, 29, 3497–3502 (1973)
[7] Beaven, G H, and White, J. C., *Nature*, 173, 389–391 (1954).
[8] Itano, H. A., Hosokawa, K, and Hirota, K., *Br J. Haemat.* (in the press).
[9] Harley, J D, and Mauer, A. M, *Blood*, 16, 1722–1735 (1960)
[10] Perutz, M F., and Lehmann, H., *Nature*, 219, 902–909 (1968)
[11] Carrell, R. W, and Lehmann, H, *Semin. Hematol.*, 6, 116–132 (1969).
[12] Rostorfer, H. H, and Cormier, M. J., *Archs Biochem. Biophys.*, 71, 235–249 (1957).
[13] Cohen, G., and Hochstein, P, *Biochemistry*, 3, 895–900 (1964).
[14] Goldberg, B, and Stern, A., *J. biol. Chem*, 250, 2401–2403 (1975)
[15] Lemberg, R., and Legge, J W, *Aust. J exp. Biol med. Sci.*, 20, 65–68 (1942).
[16] Kiese, M, and Seipelt, L, *Naunyn-Schmiedebergs Arch exp Path. Pharmak*, 200, 648–683 (1943)
[17] Allen, D W, and Jandl, J. H, *J. clin Invest.*, 40, 454–475 (1961).
[18] Jacob, H S, Brain M C., Dacie, J. W., Carrell, R W, and Lehmann, H, *Nature*, 218, 1214–1217 (1968)

Role of hybrid tetramer formation in gelation of haemoglobin S

THE transformation of erythrocytes containing haemoglobin (Hb) S from pliable biconcave disks into rigid pointed "sickled" forms, which underlies the pathology of sickle cell disease, results from the tendency of this mutant haemoglobin to polymerise and produce an intracellular gel on deoxygenation. In the genetic variants of sickle cell disease, the different types and proportions of non-S haemoglobins present in the red cells together with Hb S are important in determining the extent of the sickling tendency and the severity of the disease. Red cells containing Hb S along with a substantial proportion of foetal haemoglobin (Hb F, $\alpha_2\gamma_2$) sickle less readily than cells having a similar proportion of the normal adult type, Hb A ($\alpha_2\beta_2$). Such differences can be demonstrated with solutions of haemoglobin mixtures *in vitro* by measuring the minimum gelling concentrations (MGC), that is, the minimum concentration of total haemoglobin at which the mixture will form a gel on complete deoxygenation. The higher MGC of Hb S–F mixtures compared with S–A mixtures correlates with the lesser sickling tendency of the former combination within red cells.

There is growing evidence that tetramers of deoxy Hb S ($\alpha_2\beta_2^S$) aggregate as helical polymers in sickled cells and gels, but it is not known whether non-S haemoglobins can be included in these polymers, or if they play some other part in gel formation. Ultracentrifugation of deoxygenated haemoglobin mixtures indicated that Hb F was excluded from the solid phase of the gel, whereas some Hb A was included[1]. In solutions of oxyhaemoglobin, the tetramers are in a fairly

rapid dissociation equilibrium with $\alpha\beta$ dimers, and in mixtures of two haemoglobins (such as S–F or S–A) it has been shown that a large proportion of the haemoglobin exists as hybrid tetramers ($\alpha_2\beta^S\gamma$ or $\alpha_2\beta^A\beta^S$)[2]. There have been little experimental data concerning the possible role of such hybrid tetramers in sickling and gelation. We have approached this problem by comparing the gelling behaviour of mixtures of haemoglobins in which hybrid formation was allowed to occur, or was prevented by two different methods.

The first method used to prevent hybrid formation was based on the extremely slow rate of dissociation of deoxyhaemoglobin tetramers. The non-S haemoglobin (Hb A or F) was deoxygenated with N_2 gas in one flask, while in another flask, oxygen was eliminated from a solution of Hb S or Hb C_{Harlem} ($\alpha_2\beta_2^{6Val, 73Asn}$) which had been converted into the cyanmet form to prevent gelation. Although a solution once gelled could be liquified by chilling for the purpose of mixing, the non-gelling cyanmet form was chosen to avoid the possibility of partial aggregation which might prevent complete mixing. An appropriate volume of deoxyhaemoglobin A or F was then transferred anaerobically to the flask containing the oxygen-free cyanmet haemoglobin so that Hb S or C_{Harlem} comprised 40% of the final mixture. In spite of mixing, the deoxyhaemoglobin should remain almost entirely in the tetrametric state and thus be unable to hybridise. After chilling and thorough mixing, the cyanmet haemoglobin was converted to the deoxy form by anaerobic addition of sodium dithionite (one mol per mol total haem) and the MGC was determined at room temperature[3]. Controls for these experiments consisted of the same haemoglobins mixed in the oxy state to permit hybrid formation before deoxygenation and gelling.

The second method used to avoid hybridisation consisted of reacting the non-S haemoglobin component (Hb A or F) with a bifunctional cross-linking reagent, difluorodinitrophenylsulphone. Macleod and Hill have shown that the predominant reaction product contains an internal cross link between the amino termini of the two α chains[4]. After reaction, the cross-linked haemoglobin was separated from unreacted haemoglobin by gel filtration on Sephadex G-100 in 1 M $MgCl_2$; in this medium the non-cross-linked haemoglobin is fully dissociated into $\alpha\beta$ dimers whereas the cross-linked haemoglobin, fixed as tetramers, is eluted first by virtue of its higher molecular weight[4]. At the end of gelling experiments with mixtures of Hb S (or C_{Harlem}) and cross-linked haemoglobins A or F, electrofocusing in the deoxy state on polyacrylamide gels[2] confirmed the absence of hybrid tetramers. Control gelling experiments used the unreacted portion of the non-S haemoglobin eluted as the slower-moving fraction from the Sephadex column. After gelation, electrofocusing of these mixtures in the deoxy state revealed a large proportion of haemoglobin at the position of hybrid tetramers.

We studied three mixtures, Hb S+Hb A, Hb S+Hb F and Hb C_{Harlem}+Hb A. The effect of hybrid formation on gelation of mixtures of Hb A with C_{Harlem} was of special interest because of our earlier findings with this unusual mutant, in which each β chain carries both the "sickle substitution" β^{6Val}, and a second substitution, β^{73Asn}. Pure HbC_{Harlem} has a much higher MGC than Hb S, indicating that the β^{73Asn} substitution somehow inhibits gelling. Addition of Hb A, however, lowers the MGC of Hb C_{Harlem}, so that in mixtures containing 50% or more of Hb A, the MGC is equal to those of Hb S–A mixtures of equivalent proportions. Therefore, we postulated that the presence of hybrid tetramers having one normal β chain ($\alpha_2\beta^A\beta^{C\ Harlem}$) might eliminate the inhibitory effect of β^{73Asn} (refs 5 and 6).

The results of the gelling experiments are shown in Table 1. As expected, with the usual gelling procedure (in which hybrids occur) the MGC is much higher with mixtures of Hb S and F than with S–A mixtures. By contrast, when hybrid formation was prevented by either technique, the gelling points of the S–F mixtures were much lower, and equal to those of S–A mixtures. Thus the inhibitory effect of Hb F on gelation (and

Table 1 Effect of hybrid tetramer formation on the MGC of haemoglobin mixtures

Hb composition of mixture (40:60)	Hybrids present		Hybrids absent (or minimal)	
	Hb mixed in oxy state	Hb A or F non-cross-linked control	Hb mixed in deoxy state	Hb A or F cross-linked
Hb S + Hb A	30.2	31.6	29.4	30.2
Hb S + Hb F	39.4	37.2	30 3	32.6
Hb C_H + Hb A	30 0	31.6	35.9	37.7

Haemoglobin dialysed against 0.15 M potassium phosphate, pH 7.35, deoxygenated with N_2 gas at 25 °C, sodium dithionite added, final pH of haemoglobin 7.1; mean MGC values expressed in g Hb dl^{-1}.

sickling) seems to require the formation of $\alpha_2\beta^S\gamma$ hybrids. These findings suggest that the presence of one γ chain in the tetramer is unfavourable for polymerisation. In Hb S–F mixtures, the formation of such hybrids lowers the number of Hb S tetramers which do polymerise well. Accordingly, if hybridisation is prevented, the gelling point is lower; in fact, the presence of non-hybridising Hb F tetramers seems to have an effect on gelation equal to that of Hb A.

With mixtures of Hb C_{Harlem} and Hb A, on the other hand, the effect of hybridisation was the opposite of that seen with the Hb S–F mixtures When $\alpha_2\beta^A\beta^{C\ Harlem}$ hybrids were allowed to form, the MGC was equal to that of Hb S–A mixtures, whereas preventing formation of these hybrids led to a much higher gelling point. On the basis of earlier experiments, we suggested only one of the two β^{6Val} regions of the Hb S tetramer provided an intermolecular contact in the polymer, while the other β chain probably provided other important contacts[7,8]. The present findings support this idea, and suggest that the $\alpha_2\beta^A\beta^{C\ Harlem}$ hybrids enter the polymer. The normal β chains apparently favour polymerisation more than chains having a β^{73Asn} residue.

It was surprising to find that the presence or absence of hybridisation made little or no difference in the gelling of Hb S–A mixtures. Our findings differ from those of Moffat[9], who reported slightly lower MGC values for Hb S and Hb A mixed in the oxy state than in the deoxy state. It is not clear whether this discrepancy stems from differences in experimental conditions (type of buffer, pH) or technique. One possible interpretation of our results is that, with Hb S–A mixtures, two effects of hybrid formation on gelation are counterbalanced; if we suppose that Hb S tetramers favour gelation more than SA hybrids, then when hybrid formation occurs, the effect of reducing the number of S tetramers may be balanced by the effect of increasing the total proportion of tetramers capable of polymerising (the remaining S tetramers and the SA hybrids).

The present data shed some additional light on the so-called "sparing" effect of Hb A on gelation of Hb S. Singer and Singer first pointed out that the addition of Hb A to Hb S reduced the partial concentration of Hb S required for gelation[10]. This is also found under the conditions of our experiments. The MGC of Hb S alone is about 22 g dl^{-1} while that for a 40:60 mixture of Hb S with Hb A is about 30 g dl^{-1}, so that the partial concentration of Hb S is about 12 g dl^{-1}. Our experiments demonstrate that this sparing effect of Hb A does not require the formation of $\alpha_2\beta^A\beta^S$ hybrid tetramers. Furthermore if (and only if) hybrid formation is prevented, the same sparing effect is provided by Hb F. It seems unlikely that $\alpha_2\gamma_2$ tetramers would enter the polymer more readily than $\alpha_2\beta^S\gamma$ hybrids, the formation of which inhibits gelation. Thus, non-S Hb tetramers (A or F) seem to facilitate gelling in an equal, nonspecific manner which may not involve entry into the polymers.

These results may also help interpret our earlier findings that deoxygenated mixtures of Hb S with cyanmet Hb F showed the same gelling behaviour as Hb S–A mixtures[3]. Using

techniques of electrofocusing in the deoxy state, Bunn and McDonough have recently shown that cyanmethaemoglobin fails to form stable hybrids with deoxyhaemoglobin[11]. Deoxygenation may promote dissociation of hybrids in a mixture of Hb S with cyanmet–Hb F, so that such a mixture may be comparable with the S–F mixtures in the present experiments in which hybrid formation was prevented. In that case, cyanmet Hb F or Hb A tetramers seem to facilitate gelling in mixtures with deoxy Hb S to the same extent as do deoxy–Hb A or F tetramers. As noted above, these effects need not involve entry into the helical polymers.

This work was supported by grants from the US Public Health Service and from the American Heart Association. R.L.N. is a career scientist of the Health Research Council of the City of New York.

ROBERT M. BOOKCHIN
RONALD L. NAGEL
TANIA BALAZS

Division of Hematology,
Department of Medicine,
Albert Einstein College of Medicine,
Bronx, New York 10461

[1] Bertles, J. F , Rabinowitz, R., and Dobler, J , *Science*, 169, 375–376 (1970)
[2] Bunn, H. F., in *Haemoglobin and Red Cell Structure and Function* (edit. by Brewer, G. J), 41–53 (Plenum, New York, 1972)
[3] Bookchin, R. M., and Nagel, R. L, *J. molec Biol.*, 60, 263–270 (1971)
[4] Macleod, R. M., and Hill, R. J , *J biol Chem*, 245, 4875–4879 (1970)
[5] Bookchin, R. M, Nagel, R. L, and Ranney, H. M, *J. biol Chem*, 242, 248–255 (1967).
[6] Bookchin, R. M, Nage., R. L, and Ranney, H. M., *Biochim. biophys Acta*, 221, 373–375 (1970)
[7] Bookchin, R. M, and Nagel, R L., in *Sickle Disease, diagnosis, management, education and research*, (edit. by Abramson, H., Bertles, J. F., Wethers, D. L), 140–154 (C. V Mosby Co., St. Louis, 1973)
[8] Bookchin, R. M., and Nagel, R. L, *Sem Hematol*, 11, 577–595 (1974)
[9] Moffat, K., *Science*, 185, 274–277 (1974).
[10] Singer, K., and Singer, L, *Blood*, 8, 1008–1023 (1953).
[11] Bunn, H. F , and McDonough, M , *Biochemistry*, 13, 988–993 (1974).

Primary electron acceptor complex of photosystem I in spinach chloroplasts

PHOTOSYSTEM I (PSI) is the photochemical reaction complex involved in the generation of a low potential reductant in oxygen-evolving photosynthetic organisms. The primary photochemical reaction in PSI has been identified as the photo-oxidation of a reaction centre chlorophyll complex P700 (ref. 1). This photo-oxidation occurs both at room temperature and in the frozen state at temperatures as low as 4.2 K. At cryogenic temperatures this photo-oxidation has generally been found to be irreversible[2]. Malkin and Bearden[3] showed that the electron acceptor for this photo-oxidation reaction is a bound ferredoxin. This ferredoxin has two iron–sulphur centres with very low redox potentials ($Em_{10} = -550$ and -590 mV)[4-6] It has been proposed that these centres are the primary electron acceptor of PSI[3,4]. More recent evidence suggests it may not be[7-11], and we have shown[11] that when the bound ferredoxin is chemically reduced, illumination at low temperature results in the photo-oxidation of P700 without any change in the bound ferredoxin, this oxidation is reversible. In photosynthetic bacteria a component with an EPR signal at $g = 1.82$ has been identified as the primary electron acceptor of the photochemical system[12,13] and it is possible that a similar component might be involved in chloroplast photochemical reactions. McIntosh et al.[14] obtained kinetic evidence for the presence in PSI particles of a component with an EPR signal at $g = 2.06$ and $g = 1.76$ which showed a reversible redox change on illumination of the particles at low temperature parallel to that of P700. The changes observed were small and it was not possible to obtain a spectrum of the component. Using a more highly purified PSI preparation we have now obtained strong evidence that this component is the primary electron acceptor of PSI.

Figure 1 shows the EPR spectrum of a sample of PSI

particles frozen under illumination in the presence of sodium dithionite and methyl viologen. Spectrum A shows the spectrum at 18 K and 20 mW power. In these conditions the iron–sulphur centres can be clearly seen and are completely reduced. Spectrum B shows the spectrum of the same sample at 9 K and 100 mW. In these conditions the signals due to the iron–sulphur centres of the bound ferredoxin and the methyl viologen free radical show power saturation effects. A new signal can be seen at $g = 1.76$ and $g = 1.86$. This signal is frozen in only in samples prepared in the extreme reducing conditions used for this sample. For example, little or none of this signal can be seen in samples frozen with dithionite but without methyl viologen and is not observed in samples frozen with dithionite in the dark. It is observed in photosystem I particles, and seems to be most concentrated in those samples with the highest P700 to chlorophyll ratio. We have therefore concluded that it is an electron transport carrier with an extremely low redox potential associated with PSI.
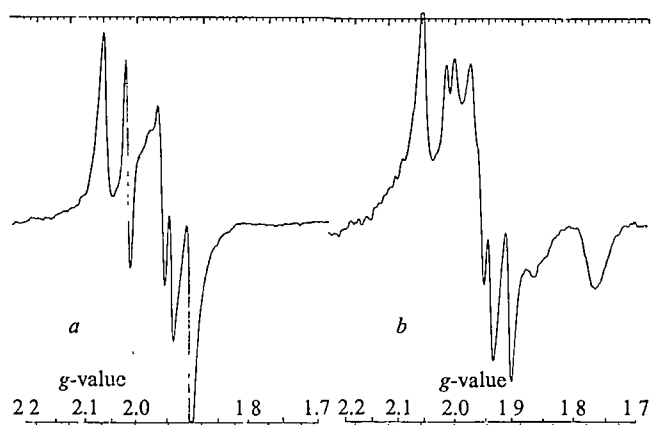
Fig. 1   EPR spectra of PSI particles showing the signal at $g = 1.76$ due to the primary electron acceptor of PSI. Highly purified particles were prepared from broken spinach chloroplasts[15] by Triton treatment[16] followed by DEAE chromatography. The sample was reduced by illumination at room temperature in the presence of 0.2% $Na_2S_2O_4$ and 15 μM methyl viologen. Chlorophyll concentration 1.5 mg ml^{-1}. EPR spectra were recorded as described previously[4,11]. The conditions for each spectrum were as follows. a, Frequency 9.24 GHz, power 20 mW, modulation amplitude 10 G, scan rate 500 gauss min^{-1}, gain 500–temperature, 18 K. b, as for a, except that the temperature was 9 K and power 100 mW. The spectra were recorded in the dark.

The g value of this component is very similar to that of the kinetic change reported by McIntosh, $et\ al.$[13]. We have examined the effect of illumination on this signal. We have shown that in PSI particles frozen with both P700 and the bound ferredoxin in the reduced state the photo-oxidation of P700 is reversible[11]. Figure 2 shows this reversible photo-oxidation in triton PSI particles. Figure 3 shows the effect of illumination of the same sample at lower temperature (9 K) and high power. The signal at $g = 2.00$ due to P700 is, much smaller in these conditions, however it can be seen that the signal at $g = 1.76$ is induced by illumination in these conditions and disappears again when the light is turned off. The kinetics of the time course shown in Figs 2 and 3 are machine limited. They do, however, indicate that P700 photo-oxidation and the appearance of the $g = 1.76$ signal occur on illumination and are fully reversible in the dark. The kinetic measurements of McIntosh $et\ al.$ indicate that the decay kinetics of the two signals are the same. In samples in which the bound ferredoxin is oxidised before illumination, illumination in the conditions described here results in irreversible oxidation of P700 and reduction of the bound ferredoxin, little or no $g = 1.76$ signal is then observed. If the $g = 1.76$ component is frozen largely in the reduced state as in the
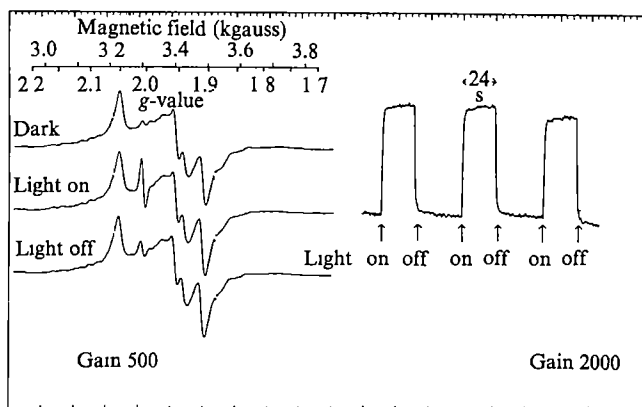
Fig. 2   The effect of illumination at low temperature on the EPR signal of P700 in Triton PSI particles. Triton PSI particles (1.5 mg chlorophyll ml^{-1}) were reduced by exposure to 0.2% $Na_2S_2O_4$ pH 9.0 for 45 min (ref. 11), before freezing to reduce the bound ferredoxins. Left, EPR spectrum in the $g = 2.00$ region. Conditions as in Fig. 1. Power 20 mW, temperature 18 K. The sample was illuminated in the spectrometer with a 1000 W projector. Right, time course of the effect of illumination on the P700 signal. Instrument settings as above except that the change in amplitude of the $g = 2.00$ signal was followed with time.

sample shown in Fig. 1 the extent of P700 photo-oxidation on illumination at cryogenic temperatures is greatly decreased. In digitonin-triton particles the $g = 1.76$ component is more enriched than in Triton particles and a signal at $g = 1.86$ is seen as well as at $g = 1.76$ on illumination. This observation together with that of McIntosh $et\ al.$[14] who observed kinetic changes around $g = 2.06$ suggest that the component has an EPR spectrum around $g = 2.00$ with g_z about 2.06, g_y about 1.86 and g_x about 1.76. This spectrum might be due to an iron–sulphur centre in a novel environment or to an unknown type of iron centre as has been proposed for the bacterial primary electron acceptor.
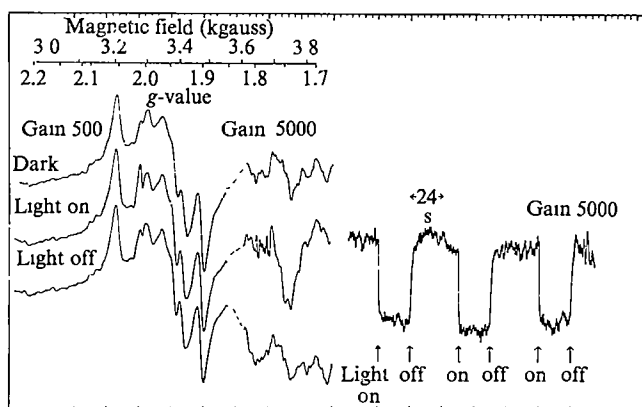
Fig. 3   The effect of illumination at low temperature on the $g = 1.76$ signal in PSI particles. The same sample was used as in Fig. 2. Left, EPR spectra in the $g = 2.00$ region. Instrument settings as in Fig. 1 with Power 100 mW, temperature 10 K. Gain as indicated. Right, time course of the effect of illumination on the $g = 1.76$ signal. Instrument settings as above except that the change in amplitude of the $g = 1.76$ signal was followed with time.

These experiments show that chloroplasts contain a redox carrier which is reduced, probably at very low potentials, in parallel with the photo-oxidation of P700. This reduction is reversible at temperatures down to 8 K. When this component is reduced P700 photo-oxidation is inhibited. When secondary electron acceptors are available P700 photo-oxidation is irreversible and this component cannot be detected. These

results suggest that the component with an EPR signal at $g = 1.76$ is the primary electron acceptor of PSI.

M. C. W. EVANS
C. K. SIHRA

Department of Botany and Microbiology,
University College, Gower Street,
London WC1E 6BT, UK

J. R. BOLTON*

Photochemistry Unit,
University of Western Ontario,
London, Ontario, Canada

R. CAMMACK

Department of Plant Sciences,
King's College, London, UK

*On leave at the Royal Institution, 21 Albemarle St., London W1X 4BS, UK

1 Kok, B., *Biochim. biophys. Acta.*, **48**, 527–533 (1961).
2 Witt, H. T., *Q. Rev. Biophys.*, **4**, 365–475 (1971).
3 Malkin, R., and Bearden, A J., *Proc natn. Acad. Sci. U.S.A.*, **68**, 16–19 (1971).
4 Evans, M. C. W., Telfer, A., and Lord, A. V., *Biochim. biophys. Acta*, **267**, 530–537 (1972).
5 Evans, M. C. W., Reeves, S. G., and Cammack, R., *FEBS Lett.*, **49**, 111–114 (1974).
6 Ke, B., Hansen, R. E., and Beinert, H., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 2941–2945 (1974).
7 Mayne, B. C., and Rubinstein, D., *Nature*, **210**, 734 (1966).
8 Warden, J. T., Mohanty, P., and Bolton, J. R., *Biochem biophys. Res. Commun.*, **59**, 822–838 (1974).
9 Visser, J. W. M., Rijgersberg, K. P., and Amesz, J., *Biochim. biophys. Acta.*, **368**, 235–246 (1974).
10 Lozier, R. H., and Butler, W. L, *Biochim. biophys. Acta.*, **333**, 460a464 (1974).
11 Evans, M C. W, and Cammack, R., *Biochem. biophys. Res. Commun.*, **63**, 1871–93 (1975).
12 Leigh, J. S., and Dutton, P. L., *Biochem. biophys. Res. Commun.*, **46**, 414–421 (1972).
13 Evans, M. C. W., Lord, A. V., and Reeves, S. G., *Biochem. J.*, **138**, 177–183 (1974).
14 McIntosh, A. R., Chu, M., and Bolton, J. R., *Biochim. biophys. Acta*, **376**, 308a314 (1975).
15 Whatley, F. R., and Arnon, D I., in *Methods in Enzymology* (edit by Colowick, S. P, and Kaplan, N. O.), **6**, 308–313 (Academic, New York, 1963).
16 Vernon, L. P., and Shaw, E. R., in *Methods in Enzymology* (edit. by San Pietro A.), **23A**, 277–288 (Academic, New York, 1971).

Genetic relationship of a primate RNA tumour virus genome to genes in normal mice

HUEBNER and Todaro[1] and Temin[2] proposed that the genomes of RNA tumour viruses originate from genes in DNA of normal cells. Subsequently, a class of RNA tumour viruses (endogenous or class 1 viruses) was discovered and shown by molecular hybridisation to contain RNA genetically related or identical to genes in normal cells[3-8]. Both the biological and hybridisation data leave little doubt that normal cells carry genes capable of giving rise to class 1 RNA tumour viruses (see refs 7 and 8 for reviews). A second class of RNA tumour viruses is more distantly related to cell genes (exogenous or class 2 viruses, see refs 7 and 8). Mouse and chicken class 2 viruses, however, are genetically related to class 1 viruses from the same species[7-12] and the genome of one class 2 mouse RNA tumour virus (Rauscher leukaemia virus) consists primarily of RNA sequences that are related distantly to genes in normal mice but not to genes found in several other animals[13]. Indirect evidence has indicated that primate RNA tumour viruses and certain mouse RNA tumour viruses are unusually closely related[10,11,14,15], suggesting that the viruses have a common origin, for example, in genes of mice. We report here that a primate RNA tumour virus isolated originally from a woolly monkey sarcoma and passaged in marmosets does contain a genome that by molecular hybridisation criteria is related to genes found in mice. The data further indicate that a small portion of the primate RNA tumour virus genome is related to genes of normal primates.

The virus analysed here, simian sarcoma virus (SiSV) or woolly monkey virus, was isolated from a natural sarcoma
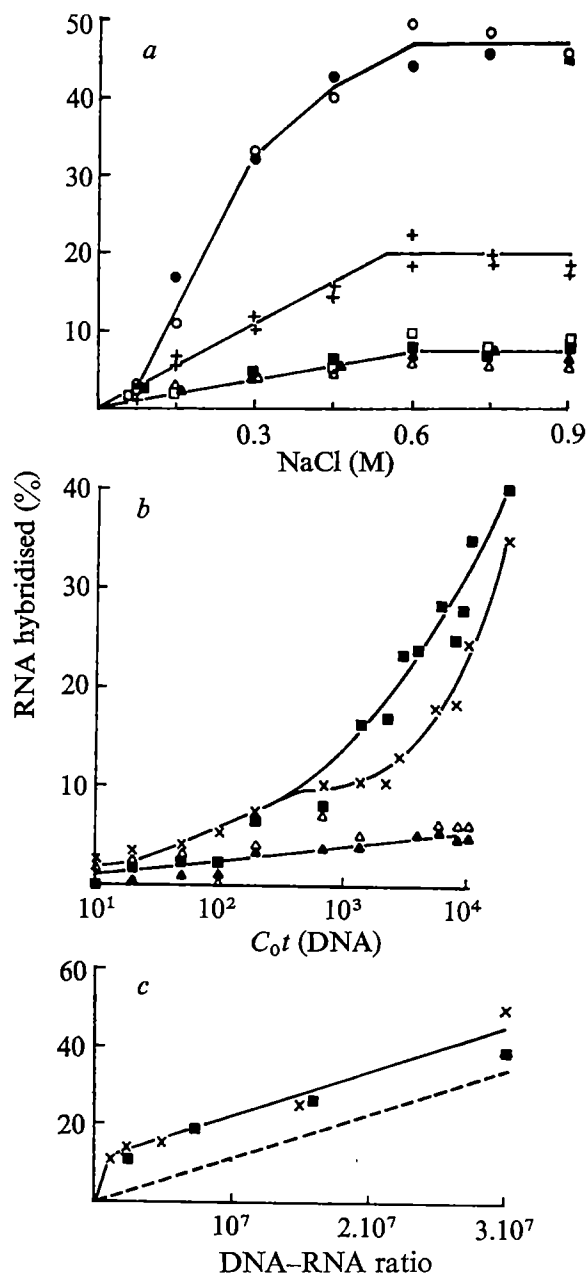
Fig. 1 Hybridisation of ^{125}I-RNA from SiSV to cell DNA. Hybridisation mixtures contained viral RNA and cell DNA (weight ratio $= 1{:}8 \times 10^6$, DNA concentration $= 8.3$ mg ml^{-1}, RNA $= 500$ c.p.m., 10^8 c.p.m. µg^{-1}) in 0.4 M PO$_4$, pH 6.8. Mixtures were boiled for 5 min, then incubated at 60 °C. *a*, Hybridisation mixtures (5 µl) were incubated for 100 h (C_0t (DNA) $= 10^4$), then exposed to 20 µg ml^{-1} RNase at 37 °C for 120 min in the indicated concentration of NaCl. Acid-precipitable radioactivity was used to measure the percentage RNA hybridised[4,13]. *b*, Hybridisation mixtures were incubated for different lengths of time, then exposed for 60 min at 37 °C to 20 µg ml^{-1} RNase in 0.6 M NaCl. *c*, Hybridisation mixtures containing different DNA-RNA ratios (DNA $= 10$ mg ml^{-1}) were incubated for 160 h (C_0t (DNA) $= 2 \times 10^4$) then exposed for 60 min at 37 °C to 20 µg ml^{-1} RNase in 0.6 M NaCl. Dashed line indicates hybrid yield as a function of the ratio DNA-RNA, subtracting the contribution by rapidly-annealing DNA sequences. This function was subjected to a double reciprocal analysis (1/hybrid yield against RNA-DNA ratio) and yielded an x-intercept value of 0.022 (45% RNA hybridised). This is taken to reflect the percentage RNA that could be hybridised to infrequent DNA sequences at infinite DNA input. Percentage RNA that could complex repeated plus infrequent DNA sequences at infinite DNA input is therefore 56. Sources of cell DNA: *a*: ○, 7IAPI (SiSV); NRK (SiSV); +, mouse; □, gibbon; ■ woolly monkey; ▲, chimp; △, human; *b* and *c*: ■, HF (SiSV); ×, mouse; △, woolley monkey; ▲, human. Less than 25% of the acid-precipitable radioactivity was lost from the RNA after 160 h of incubation at 60°. RNase-resistant radioactivity obtained without DNA are subtracted from each hybrid yield value (3–5% of the input radioactivity).

of a woolly monkey[16]. The monkey tumour was homogenised, and a cell-free extract was placed on normal marmoset lung cells (line 1283), which then began to produce virus. The virus-producing cells were inoculated into a marmoset (71AP1) causing the development of a tumour which was explanted and grown in culture. The tumour cells produced a virus, referred to here as SiSV (71AP1). To the best of available knowledge, none of the biological materials, save possibly the original woolly monkey itself, had direct contact with rodents or rodent cells. SiSV (71AP1) has subsequently been introduced into several other cell lines, including marmoset HF cells and normal rat kidney NRK cells.

The experiments reported here involve hybridisation of 70S RNA isolated from SiSV (71AP1) and labelled with ^{125}I *in vitro*[17] to DNA purified from a variety of animal tissues or cultured cell lines. As the objective was to examine genes of normal cells for sequences related but not necessarily identical to the viral RNA, conditions of hybrid formation and detection capable of monitoring imperfect RNA–DNA complexes was required. Hybridisation of viral RNA to cell DNA at 60 °C in 0.4 M PO_4 has been found to be a suitable condition of hybrid formation for this purpose[13]. The condition of hybrid detection most suitable for detecting imperfect complexes formed between SiSV (71AP1) RNA and cell DNA (8×10^6-fold weight excess) was treatment of the hybridisation mixture with 20 μg ml^{-1} RNase A in 0.6 M NaCl (Fig. 1a). The experiments used a 120 min exposure of the hybrid mixture to RNase. In these conditions, RNA from SiSV (71AP1) formed RNase-resistant complexes with DNA from rat NRK or marmoset 71AP1 cells infected with SiSV (50% of the RNA) and with DNA from normal mice (20% of the RNA). The RNA–DNA hybrids were maximally protected from degradation by RNase in 0.6 M NaCl; some complexes were destroyed in solutions containing less NaCl. Hybrid yield using DNA from uninfected primates was only 5–10% of the RNA when RNase treatment was carried out for 120 min (Fig. 1a), but was as high as 20–25% when RNase treatment was limited to 60 min (Table 1).

Table 1 presents hybridisation results for ^{125}I RNA from SiSV (71AP1) to DNA from many animals, using a single condition of hybrid formation (60 °C, 0 4 M PO_4, $C_0t = 10^4$) and hybrid detection (RNase treatment in 0.6 M NaCl at 37 °C for 60 min). DNA from mice or rats hybridised more SiSV RNA than did DNA from other animals, but appreciable hybridisation was obtained with DNA from some Old World primates and, to a lesser extent, from New World monkeys. DNA from chimpanzees hybridised 25% of the SiSV RNA, whereas DNA from humans hybridised only 5–10%. DNA from the lower animals examined, excluding mice and rats, hybridised only 1–10% of the RNA.

The kinetics of hybridisation of SiSV (71AP1) RNA to DNA from NIH Swiss mice or from SiSV-infected marmoset HF cells was that expected from DNA sequences present only a small number of times per genome (Fig. 1b). By a C_0t of 2×10^4, 35% of the RNA had hybridised to DNA from mice and there was no indication that the hybridisation reaction was slowing. A small portion of the RNA (10%) annealed rapidly to DNA from mice or infected marmoset HF cells. DNA from humans or woolly monkeys reacted poorly with SiSV RNA, even at high C_0t. The t_m in 0.15 M NaCl of the hybrid structures formed with DNA from mice or chimpanzees was only about 65 °C, compared with 80 °C for hybrids involving DNA from SiSV-infected marmoset 71AP1 cells. Therefore, the virus-related sequences in the DNA of mice and chimpanzees are less closely related to the viral genome than are the proviral DNA sequences in the DNA of SiSV-infected cells.

The DNA–RNA ratio in the experiments described above was 8×10^6. If each viral subunit has a molecular weight of

Table 1 Hybridisation of RNA from SiSV to DNA from various animals

DNA source	Hybrid yield (% RNA hybridised)
Mice, rats and infected cells	
71AP1 (SiSV) (C)	55
NIH Swiss mouse (S, L)	25
A31 mouse (C)	23
Wild-type mouse (S, L)	15
MOPC mouse (Tu)	22
NRK normal rat kidney (C)	16
Old World primates	
Chimpanzee (M)	25
Gibbon (B)	20
Celebese ape (M)	21
Hamadrayas baboon (T)	15
Mandrill baboon (B)	16
Slow loris (M)	15
Grivet (M)	6
Vervet (T)	9
Crab-eating macaque	7
Stumptail macaque	8
Human (WBC)	5
New World primates	
Capuchin (T)	18
Marmoset (L)	12
Owl monkey (T)	11
Spider monkey (M)	11
Howler monkey (M)	11
Woolly monkey (S)	11
Squirrel monkey (S)	7
Other animals	
Pig (L)	10
Squirrel (S, L)	8
Cat (S, L)	8
Chicken (E)	4
Racoon (S, L)	2
Slug (A)	1

RNA–DNA hybridisation mixtures were constructed as described in the legend to Fig. 1a, then exposed to 20 μg ml^{-1} RNase at 37 °C for 60 min in 0.6 M NaCl. Letters in parentheses indicate the tissue or cell type used to obtain DNA. C, Cultured cells; S, spleen; L, liver; Tu, tumour; M, muscle; B, brain; T, thymus; E, embryo; A, whole animal; WBC, White blood cells.

3×10^6 (ref. 18) and if the viral-related sequences exist only once per genome, complementary DNA should be 27-fold in excess. If the viral-related sequences in the mouse DNA, however, are only distantly related to RNA from SiSV (71AP1), after hybridisation the RNA–DNA hybrids can exist in reversible equilibrium with free RNA. Raising the amount of DNA would then drive more RNA into RNA–DNA hybrids. Figure 1c shows that at a C_0t of 2×10^4, increasing the DNA–RNA ratio to 30×10^6, resulted in hybridisation of 45% of the SiSV (71AP1) RNA with normal mouse DNA—a hybrid yield equivalent to that obtained with DNA from SiSV-infected marmoset HF cells. Again there was indication that 10–11% of the RNA hybridised to repeated DNA sequences. These data when analysed by a double reciprocal representation showed that 56% of the SISV RNA would have hybridised to mouse DNA at infinite DNA input (see legend to Fig. 1).

Several reports[14] have suggested an unusual genetic proximity between viral sequences in primates, including man, and those in viruses from mice[10,11,15]. It has also been demonstrated that both 'endogenous' and 'exogenous' mouse RNA tumour viruses carry genomes related to DNA sequences in normal mice[7,13,19]. Taken together, these observations indicate that the primate viruses also contain genomes related to genes in normal mice, a conclusion verified by our studies. The concept of mouse–primate relationship of RNA tumour virus nucleotide sequences is supported by the isolation from cultured asian mouse cells of a virus containing proteins antigenically related to analogous proteins from primate RNA tumour viruses[20].

The homology between SiSV RNA and normal mouse

DNA can be used as an indication that SiSV originated in mice and subsequently entered primates. The weaker homology between SiSV RNA and primate DNA may reflect genetic change promoted by recombination with the new host[2]. The hybridisation results show a closer homology of the virus to Old World monkeys than to New World monkeys, indicating genetic interaction of the virus with Old World monkeys. SiSV (71AP1) was isolated from and has subsequently had contact solely with New World monkeys or cells derived from New World monkeys. We have been told by the owner of the woolly monkey from which SiSV was obtained that this household pet cohabited for 1–2 years with a gibbon before the woolly monkey developed a sarcoma. Possibly, the gibbon carried the virus and transmitted it to the woolly monkey. The possibility of an Old World primate transmitter of a mouse virus as a means for generating primate RNA tumour viruses is being studied.

We thank S. Gillespie and F. Wells for assistance, Drs W. C. Saxinger, F. Deinhardt, R. G. Smith, R. E. Gallagher, and R. C. Ting for discussions, and especially E. Johannsen for obtaining tissues from several of the animals studied.

F. WONG-STAAL
R. C. GALLO
D. GILLESPIE

National Cancer Institute,
Laboratory of Tumor Cell Biology,
Bethesda, Maryland 20014

1 Huebner, R. J., and Todaro, G. J., *Proc. natn. Acad. Sci. U.S A.*, **64**, 1087 (1969).
2 Temin, H. M., in *Perspectives in Biology and Medicine*, 11 (Academic, New York, 1970)
3 Neiman, P., *Nature new Biol.*, **244**, 62 (1973).
4 Gillespie, D , Gillespie, S., Gallo, R. C., East, J. L , and Dmochowski, L., *Nature new Biol.*, **244**, 51 (1973).
5 Neiman, P., *Virology*, **53**, 196 (1973).
6 Nayak, D. P., and Murray, P. R., *J. Virol.*, **12**, 177 (1973).
7 Gillespie, D., and Gallo, R C., *Science*, **188**, 802 (1975).
8 Gillespie, D., Saxinger, W. C., and Gallo, R. C., *Prog. Nucl. Acid Res. molec. Biol.*, **15**, 1 (1975).
9 Neiman, P. E., Wright, S. F., McMillin, C., and MacDonnell, J. *Virol.*, **13**, 837 (1973).
10 Benveniste, R. E., and Todaro, G. J., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 3316 (1973).
11 Miller, N., *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 3177 (1974).
12 Okabe, H., Gilden, R. V., and Hatanaka, M., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 3293 (1973).
13 Gillespie, D., Gillespie, S., and Wong-Staal, F., in *Meth. Cancer Res.*, 11 (in the press).
14 Hehlmann, R., Kufe, D., and Spiegelman, S., *Proc. natn. Acad. Sci. U.S.A.*, **69**, 435 (1972).
15 Gallo, R. C, Miller, N. R., Saxinger, W. C., and Gillespie, D., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 3219 (1973).
16 Wolfe, L. G., *et. al.*, *J. Natn. Cancer Inst.*, **47**, 1115 (1971).
17 Prensky, W. D, Steffensen, D. M., and Hughes, W. L., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 1860 (1973).
18 Duesberg, P. H., *Proc. natn. Acad Sci. U.S.A.*, **60**, 1511 (1968).
19 Stephensen, J. R., and Aaronson, S. A., *J. Virol.*, **46**, 480 (1971).
20 Lieber, M. M., *et al.*, *Proc. natn. Acad. Sci. U.S.A.* (in the press).

Specific binding of CAP factor to *lac* promoter DNA

CATABOLITE repression in *Escherichia coli* is mediated by the cyclic AMP dependent catabolite activator protein (CAP). Growth conditions where the induced catabolism of certain sugars is energetically favourable, primarily in the absence of glucose, promote the intracellular induction of a high cyclic AMP concentration; CAP binds cyclic AMP, is activated, and stimulates mRNA synthesis from promoters of catabolite repressible operons[1–4]. The mechanism by which CAP influences the interaction between RNA polymerase and promoter DNA in unknown. Purified CAP preparations exhibit a cyclic AMP-dependent DNA binding activity, but efforts to detect specific binding to promoters of catabolite repressible genes have been unsuccessful; all DNAs tested have shown similar binding affinities[5,6]. The only evidence for a specific interaction between CAP and promoter DNA is a fluorescent probe study which showed that a *lac* transducing phage DNA molecule induces a conformational change in CAP that is not seen with the wild-type phage molecule[7]; but no

effort was made to correlate this difference with *in vivo* CAP behaviour. Here I report experiments which demonstrate that the CAP factor binds specifically to a site within the *lac* promoter. This binding is sensitive to mutations which affect the ability of CAP to stimulate *in vivo* and *in vitro lac* gene expression.

These experiments were made possible by the recent isolation of a 200 base pair DNA fragment containing an intact *lac* control region[8]. The fragment, designated *Hae*200, was generated by digesting 1,000 base pair sonicated *lac* fragments with the *Hae*III restriction endonuclease of *Haemophilus aegyptius*[9] and was shown to contain the entire control region by the following criteria: first, an *in vitro* transcription system using *Hae*200 as a template synthesises mRNA which extends into the region coding for the NH_2 terminus of β-galactosidase, the product of the *z* gene (J. M., unpublished). Second, polyacrylamide gel electrophoresis of a digest of DNA from a strain containing the L1 deletion, known to cover both the COOH-terminus of the *i* gene and part of the *lac* promoter[10,11], shows that only the mobility of the *Hae*200 fragment is altered by the deletion[8]. Therefore the entire region from the end of the *i* gene to the beginning of the *z* gene must be on the fragment. Figure 1 shows a map of the *lac* region and the fragments generated by *Hae*III digestion.

I have investigated the ability of CAP to bind these fragments to nitrocellulose filters; details of the binding conditions are given in the legend to Fig. 2. The CAP preparation used in these assays, when used in an *in vitro* transcription system, will stimulate mRNA synthesis off the *Hae*200 fragment up to twentyfold. This stimulation is dependent on cyclic AMP and saturates at CAP concentrations similar to the maximum concentrations used in the binding assays (J. M., unpublished). All binding was carried out in the presence of a large excess of unlabelled, sonicated λ*plac*$_5$ DNA fragments; in the absence of these fragments no specific binding could be detected. Figure 2 displays the DNA binding activity of CAP towards the *Hae*200, *Hae*175, and *Hae*120 fragments. In these conditions CAP shows significantly greater affinity for the promoter containing *Hae*200 fragment than for either of the other fragments. The binding is dependent on cyclic AMP and can be inhibited by cyclic GMP, a competitive inhibitor of cyclic AMP[1]. At higher concentrations of CAP the specificity becomes considerably less dramatic as the effect reported here is dependent on both the absolute CAP concentration and the CAP–DNA ratio.

If the affinity for *Hae*200 is functional it should be affected by promoter mutations which alter the ability of CAP to stimulate *lac* transcription. A suitable test for this is the CAP independent double mutant L8-UV5. L8, probably a point mutation, maps under the L1 deletion and gives a fifteenfold reduction in CAP stimulation[10–12]. UV5 is a second mutation which reverts L8 to a CAP independent, *lac*+ phenotype[13]. Figure 3 shows that the affinity of CAP for *Hae*200 from L8-UV5 is significantly less than that for the wild-type frag-

Fig. 1 Map of the *lac* control region with the location of *Hae*III cuts as determined by Gilbert *et al.*[8]. Regions of interest on *Hae*200 are given in more detail, as determined by Gilbert *et al.*[16], Dickson *et al.*[14] and Beckwith *et al.*[13].

*Hae*III fragments
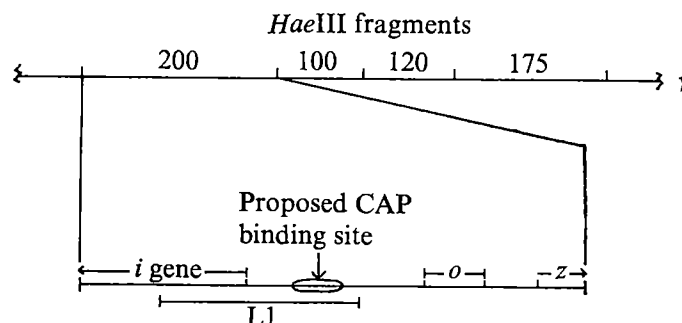
ment, especially at low CAP concentrations; although the *Hae*175 fragments from the same preparations of the two strains bind with equal affinity (see also Fig. 2). The L1 deletion itself completely eliminates the ability of CAP to influence *lac* transcription[10,11]. Figure 3 shows that the fragment corresponding to *Hae*200 isolated from the L1 strain (about 80 base pairs shorter than that from wild type) also shows little affinity for CAP. Similar effects have been seen with CAP preparations
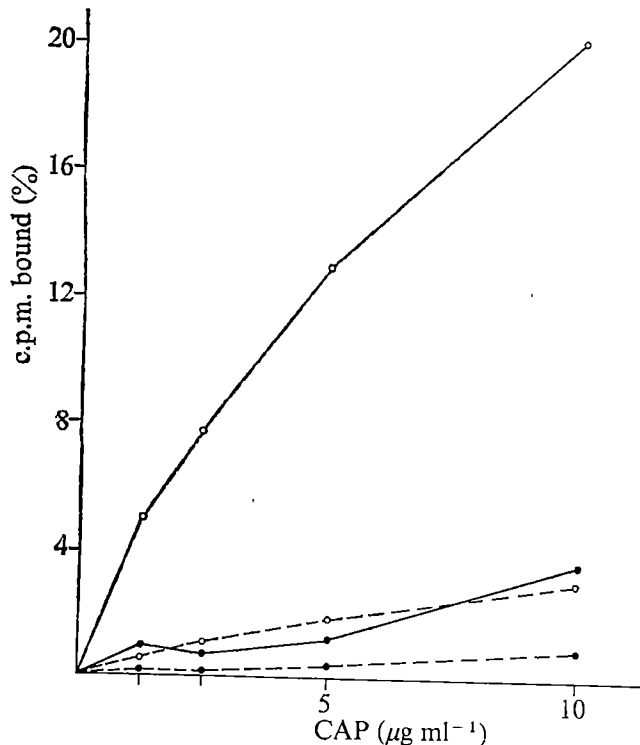
Fig. 2 Filter binding affinity of CAP for the *Hae*200, *Hae*175, and *Hae*120 fragments. Each binding reaction contains in 200 µl: 20 mM Tris-HCl pH 8.0, 10 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM cyclic AMP or cyclic GMP, 100 µg ml^{-1} bovine serum albumin, 10 µg ml^{-1} sonicated λ*plac₅* fragments, and ^{32}P-labelled restriction fragments, from 1,000 to 5,000 c.p.m. per assay. Labelled fragments were purified as described by Gilbert and Maxam[17], the specific activity of the fragments was about 10 Ci per mM phosphate The fragments, dialysed into 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA, were made up to 10 mM KCl, 10 mM $MgCl_2$, 0 1 mM dithiothreitol and 200 µg ml^{-1} BSA. 10 µg of carrier, sonicated 1,000 base pair fragments were added along with enough *Hae*III enzyme to yield a complete digest after an incubation of one hour at 37 °C. The digested fragments were made up to 2 M NH_4OAc and precipitated with two volumes of ice-cold 95% ethanol They were resuspended in 0.5 M NH_4OAc, again ethanol precipitated, then electrophoresed through a 5% polyacrylamide TBM (0.1 M Tris-borate pH 8.3, 2 mM $MgCl_2$) gel[17]. The radioactive bands, identified by autoradiography, were cut out and eluted into 0 5 ml of 0.5 M NH_4OAc, 10 mM $Mg(OAc)_2$, 0.1 mM EDTA, 0.1% SDS, containing 20 µg of tRNA. The DNA was then ethanol precipitated, resuspended in 0.5 M NH_4OAc, reprecipitated, and stored in 100 µl of 10 mM Tris-HCl pH 7.5. CAP was purified by the method of Pastan *et al.*[18], modified by the omission of dithiothreitol throughout. The purified protein was dialysed into 20 mM Tris-HCl pH 8.0, 50 mM KCl and stored at 4 °C For the binding assay, salts, cyclic AMP, CAP, DNA fragments and BSA were incubated for 10 min at room temperature in silicated glass tubes, then filtered through S & S B-6 nitrocellulose filters that had been presoaked in 0.1 N NaOH, washed thoroughly in double distilled water and equilibrated with binding buffer. The filters were then washed twice with 0.15 ml binding buffer without BSA, dried thoroughly, and counted in toluene Omnifluor. Backgrounds in which no CAP was added have been subtracted in all cases. Typically these were between 5 and 10% of the input counts. Those cases in which bound counts were below background have been recorded as 0% counts bound. O————O, Wild-type *Hae*200+cyclic AMP; ●————●, wild-type *Hae*200+cyclic GMP; O————O, wild-type *Hae*175+cyclic AMP; ●————●, wild-type *Hae*120+cyclic AMP.
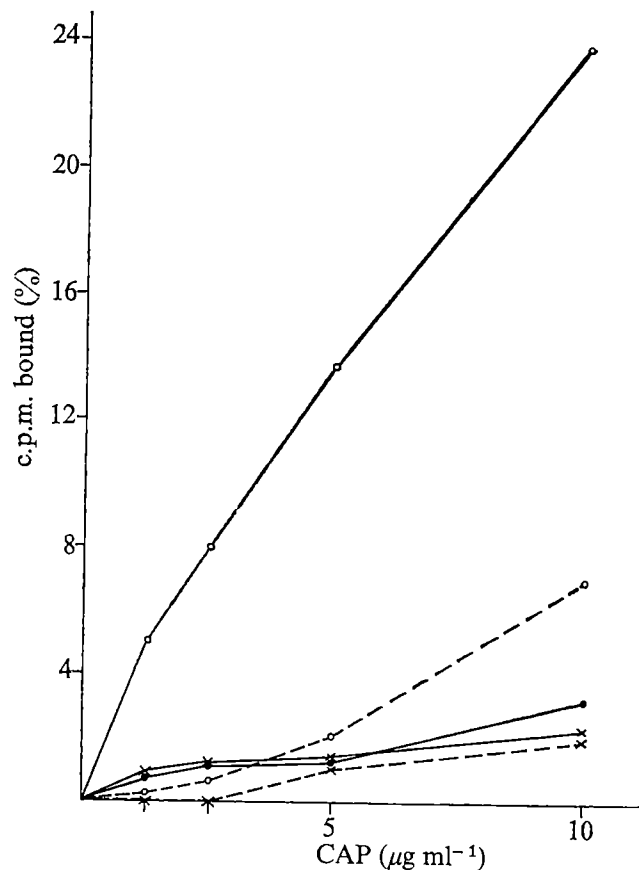
Fig. 3 *a*, Comparison of the binding of wild-type *Hae*200 to that of two mutants, L8-UV5 and L1. Conditions are the same as described in the legend to Fig. 2. 0.25 mM cyclic AMP was present in each assay. O————O, Wild-type *Hae*200; O————O, L8-UV5 *Hae*200; ●————●, L8-UV5 *Hae*175; ×————×, L1 *Hae*200; ×————×, L1 *Hae*175.

purified in different ways and with several DNA preparations. I conclude that the observed DNA binding activity is functional and must be involved in the CAP regulatory function.

As for the mechanism of this regulation, the *lac* promoter sequence of Dickson *et al.*[14] reveals that the L1 deletion covers a symmetry region of about 16 bases which may be the CAP binding site. This site is about 10 base pairs removed from a region to the right of the L1 deletion in which several mutations have been mapped and sequenced which reduce the affinity of the polymerase for the promoter in a *crp*⁻ background[14,15]. The polymerase probably recognises the DNA sequence in this mutatable region and this recognition event is a likely candidate for CAP regulation. This regulation may be effected either by a change in the conformation of the DNA, in which case there would be no direct CAP–polymerase interaction, or by a direct stabilisation through a CAP–polymerase–DNA complex. Although CAP has not been shown to interact directly with the RNA polymerase, conformational changes induced by promoter DNA sequence information may make possible such an interaction. Alternatively, the direct protein–protein interaction needed (about 2–3 kcalorie since CAP enhances the promotion by a factor of fiftyfold *in vivo*) may be too weak to have been detected by the methods used so far.

I thank Walter Gilbert for discussions and for help with the manuscript. This work was supported by a Public Health Service grant from the National Institute of General Medical Sciences.

JOHN MAJORS

Committee on Biophysics,
Harvard Biological Laboratories,
Harvard University,
Cambridge, Massachusetts 02138

¹ Zubay, G , Schwartz, D., and Beckwith, J., *Proc. natn. Acad. Sci. U.S.A.*, **66**, 104–110 (1970).

[2] Emmer, M , deCrombrugghe, B., Gottesman, M., Perlman, R., and Pastan, I., *Proc. natn. Acad Sci. U.S.A.*, **66**, 480–487 (1970).

[3] Nissley, S., Anderson, W., Gottesman, M., Perlman, R , and Pastan, I., *J. biol. Chem.*, **246**, 4671–4678 (1971)

[4] deCrombrugghe, B , *et al* , *J. biol. Chem* , **246**, 7343–7348 (1971).

[5] Riggs, A., Reines, G., and Zubay, G., *Proc. natn Acad. Sci. U.S.A.*, **68**, 1222–1225 (1971).

[6] Nissley, P., Anderson, W., Gallo, M., Pastan, I., and Perlman, R , *J biol. Chem.*, **247**, 4264–4269 (1972).

[7] Wu, F., Nath, K., and Wu, C. -W , *Biochemistry*, **13**, 2567–2572 (1974).

[8] Gilbert, W., Gralla, J., Majors, J , and Maxam, A , in *Protein–Ligand Interactions*, (edit. by Sund, H., and Blauer, G.), 193–210 (Walter deGruyter, Berlin, 1975)

[9] Middleton, J , Edgell, M , and Hutchison, C , III, *J Virol* , **10**, 42–50 (1972).

[10] Scaife, J , and Beckwith, J., *Cold Spring Harb. Symp. quant. Biol.*, **31**, 403–408 (1966)

[11] Miller, J., Ippen, K., Scaife, J., and Beckwith, J , *J. molec. Biol.*, **38**, 413–420 (1968).

[12] Beckwith, J., Grodzicker, T , and Arditti, R., *J. molec. Biol.*, **69**, 155–160 (1972).

[13] Silverstone, A., Arditti, R., and Magasanik, B., *Proc. natn. Acad Sci. U.S A.*, **66**, 773–779 (1970).

[14] Dickson, R., Abelson, J , Barnes, W., and Reznikoff, W., *Science*, **187**, 27–35 (1975).

[15] Hopkins, J., *J. molec. Biol* , **87**, 715–724 (1974).

[16] Gilbert, W., Maizels, N., and Maxam, A., *Cold Spring Harb. Symp. quant Biol* , **38**, 845–855 (1973).

[17] Gilbert, W., and Maxam, A., *Proc. natn. Acad. Sci. U.S.A.*, **70**, 3581–3584 (1973).

[18] Pastan, I., Gallo, M., and Anderson, W., in *Methods in Enzymology*, **38**, (edit. by Hardman, J., and O'Malley, B.), 367–376 (Academic, New York, 1974).

Translation of plant messengers in egg cells of *Xenopus laevis*

MAMMALIAN and plant virus messengers can be translated in living egg cells and oocytes of the tadpole of *Xenopus laevis*[1-6], and this method could be useful in the study of messenger

Time (h)
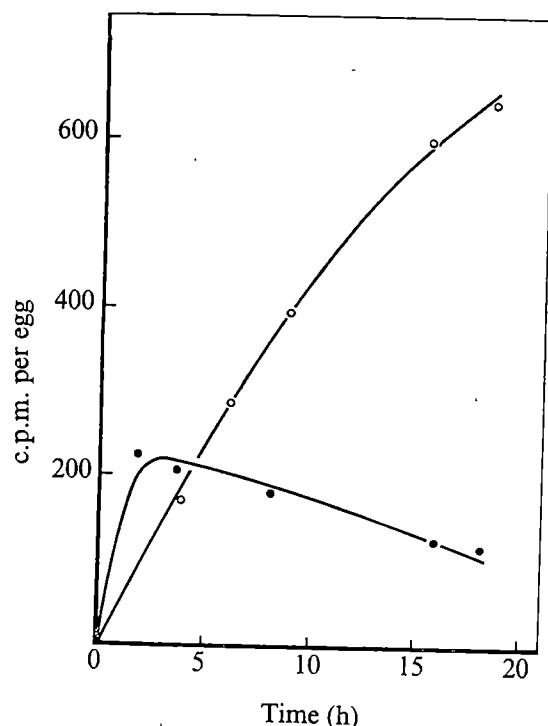
Fig. 1 The effect of injection of crude plant RNA extract on the synthesis acid precipitable material by egg cells of *Xenopus laevis*. The RNA was extracted from self-pollinated styles of *Petunia hybrida* using the cold phenol method[7]; 20–30 ng RNA was injected per egg together with 0.25 µCi ³H-leucine (specific activity 32 Ci mmol⁻¹) in a total volume of 100 nl injection medium[1]. In the control experiments the same amount of radioactivity was injected also in 100 nl. Injection was carried out with a microinjection device with which nanolitre quantities could be injected without precalibration[11]. After incubation during the indicated times in saline solution[1] containing 1 mg actinomycin D l⁻¹, eggs were homogenised in 0.5 ml of a buffer containing 0.05 M Tris-HCl(pH, 7.6), 5 mM Mg acetate, 25 mM NH₄Cl, 6 mM β-mercaptoethanol and 0.2% SDS in a Braun homogeniser, and finally taken up in 2 ml of this buffer. After centrifugation at 2,500g at 4 °C an equal volume of 20% TCA was added to the supernatants and after 30 min at 0 °C the precipitate was centrifuged down at 30,000g. The pellet was re-suspended in 10% TCA, heated at 90 °C for 15 min, cooled and again centrifuged at 30,000g. The pellet was resuspended in 0.1 ml of a proper buffer per egg cell; 10 µl was counted in a toluene-based scintillation liquid in a Philips Liquid Scintillation Counter. ●, No RNA injected; O, 20–30 ng total RNA extract injected per egg cell.

activity in general. Here I describe the results of experiments aimed at the use of this method for studying plant messenger RNA.

During the incompatibility reaction in *Petunia hybrida*, the style reacts to self and cross pollination by differential gene activity as measured by the synthesis of RNA[7]. Since these differences were most likely to be observed in messenger RNA, protein synthesis was also studied. Polysomes extracted from pollinated styles were incubated *in vitro* with a 105,000*g* rat liver supernatant[8]. The quantitative patterns of protein and RNA synthesis were very similar in the time after pollination[8]. Qualitative analysis of the newly synthesised polypeptides by means of electrophoresis, however, with or without SDS and urea, and isoelectric focusing, always showed one broad dominant peak and very little incorporation in other parts of the gel. These results did not agree with those obtained for RNA synthesis[9]. Nevertheless, this *in vitro* system demonstrated that it was possible to use animal enzymes to study the translation of plant messengers. It was therefore decided to investigate the possibility of using *Xenopus* egg cells for this purpose. Unfertilised egg cells could be collected the morning after injection of a female with hormone[10]. The egg cells were treated according to the procedures described by Gurdon *et al.*[1] except for the addition of 1 mg actinomycin D per l incubation medium. The effect of injection of total RNA extract from self-pollinated styles is given in Fig 1. At first the plant RNA tends to inhibit the translation of endogenous RNA but subsequently its translation occurs at an almost constant rate for several hours.

To prove that the level of incorporation is proportional to the amount of mRNA injected into the cell and depends exclusively on mRNA, a total cold phenol extract from five self-pollinated styles was electrophoretically separated in a tandem polyacrylamide gel[6]. The gel was then cut into five parts, comprising the RNA with sedimentation values of 28S and more, between 18S and 28S, 18S, between 5S and 18S, and 5S or less. Equal amounts of the re-extracted RNA were injected into *Xenopus* eggs and the radioactivity in the acid-precipitable material was measured (Fig. 2).

Fig. 2 The stimulation of incorporation of ³H-leucine by different RNA fractions. A crude RNA extract from 10 styles was separated electrophoretically in polyacrylamide tandem gels[7]. After electrophoresis the gels were cut into five parts. The corresponding parts were homogenised in such a volume of double concentrated injection medium that the concentration of RNA in all fractions was about 20 ng per 50 nl. The gel slurry was centrifuged at 10,000*g* for 10 min at 4 °C and the supernatants collected. Aliquots were diluted 1:1 with ³H-leucine and 100 nl of this mixture was injected into each egg cell. Incubation, extraction and measurement of radioactivity were as described in Fig. 1. Solid line; absorbance trace of one gel. Hatched blocks; Incorporation of ³H-leucine per µg RNA.
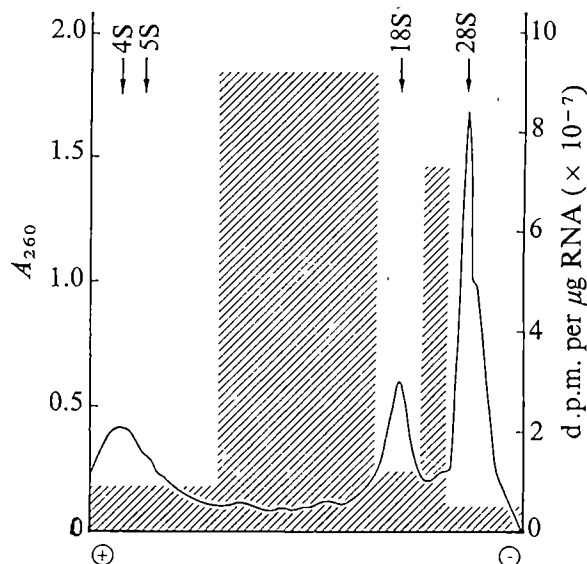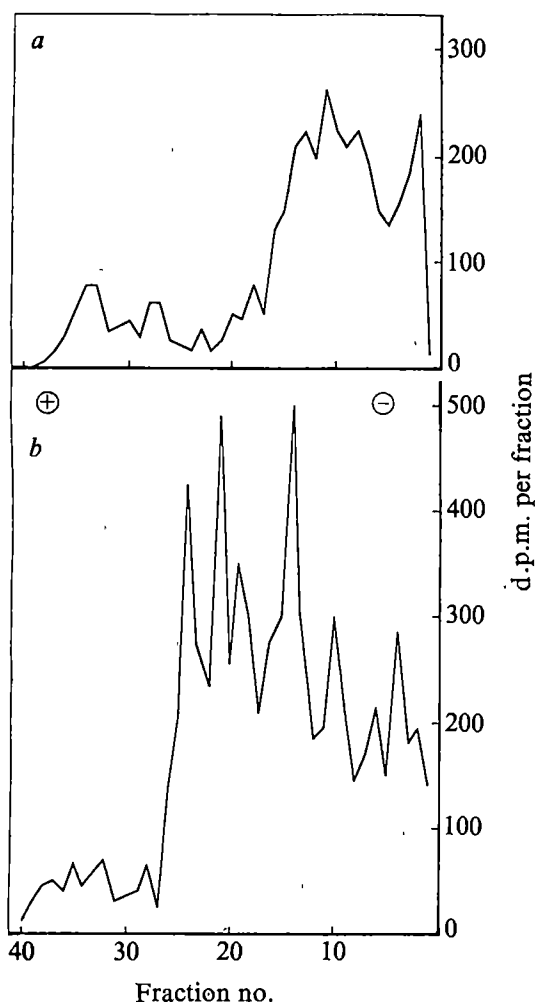
Fig. 3 Distribution of radioactivity after electrophoresis of protein extracts from treated *Xenopus* egg cells. Electrophoresis was carried out in the lower gel of 13.5% polyacrylamide with a stacking gel of 2.7% polyacrylamide at 3 mA per gel and 70 V in the presence of 4 M urea at pH 8.3 (0.05 M Tris-glycine buffer containing 0.2% SDS). Electrophoresis was stopped when cytochrome c had migrated 25 mm in the lower gel. The stacking gel was removed and counted as fraction number 1. The lower gel was cut into 1-mm slices (Mickle Gel Slicer). Gel slices were combusted (Packard Sample Oxidyser) and counted. *a*, Only endogenous RNA is translated; *b*, 25 ng of total RNA extract injected per egg cell.

Although most of the RNA was recovered as ribosomal and transfer RNA, the highest incorporation levels per mg RNA were obtained after injection of RNA from the parts of the gel in between those peaks. The conclusion, therefore, has to be that incorporation of ^{3}H-leucine only depends on the amount of messenger injected. The most difficult part of this study, however, is to prove that the proteins synthesised in *Xenopus* after injection of plant RNA contain plant proteins. After electrophoretical separation in SDS–urea–polyacrylamide gel the patterns of distribution of label were different for proteins synthesised in either the presence or absence of total plant RNA extract (Fig. 3). Immunological proof of identity will be conclusive when only one messenger is used and the protein synthesised this way has antigen activity. When a complete RNA extract is used, however, the method becomes questionable, especially since many plant proteins have no antigenic activity. In this special case, however, RNA was extracted from self-pollinated self-incompatible styles. Thus it could be assumed that part of the proteins translated from it were involved in the incompatibility reaction, that is the inhibition of the pollen tube growth in the style. Therefore, if the right plant proteins were synthesised in *Xenopus*, application of these proteins to cross-pollinated styles would have the same effect on pollen tube length as a protein extract from self-pollinated styles, whereas

Table 1 The effect of proteins synthesised in *Xenopus* eggs on pollen tube length

Pollination	Addition	% Reduction of compatible tube length
	—	0
	Water/buffer	2
	Endogenous *Xenopus* proteins	2–5
Compatible ($S_3 \times S_2$)	Protein extract of *Xenopus* after injection of RNA from $S_3 \times S_3$	30–40
	Protein extract of self-pollinated styles	20–40
Incompatible ($S_3 \times S_3$)	—	20–40

Protein samples were obtained as described in Fig. 1, except for heating at 90 °C, but dialysed against running tap water, double distilled water or electrophoresis buffer (see Fig. 3) for at least 24 h. They were subsequently applied to the style by dipping the stigma in this solution, containing 400–600 µg protein per ml as determined according to Lowry *et al.* After 30 min at room temperature the styles were pollinated as indicated and incubated at 25 °C for 24 h. Pollen tube lengths were measured by ultraviolet fluorescence microscopy[12].

proteins synthesised with endogenous *Xenopus* RNA would have no effect. The results are as listed in Table 1.

Proteins synthesised in *Xenopus* after injection of RNA extracted from cross-pollinated styles also showed an effect on pollen tube growth. But stimulation was found only when the proteins were tested in a combination of pollen and style that did not contain any of the *S* alleles present in the combination of pollen and style from which the RNA was extracted. Reduction of pollen tube length was found when these *S* alleles were identical. These results indicate that the primary gene product of the *S* allele is synthesised in the style after pollination. They also tend to show that the polypeptides are involved in the recognition reaction which is very specific. Since these polypeptides can be synthesised in *Xenopus* egg cells with plant messengers, we may conclude that the machinery for the synthesis of proteins in eukaryotic plants and animals is very similar and that this method provides a useful tool in the study of gene activity in both plants and animals.

I thank professor H. F. Linskens for his continuous interest and J. R. de Haas for providing the *Xenopus* eggs. Part of this research was made possible by financial support from the Association Euratom-ITAL.

J. A. W. M. VAN DER DONK

Department of Botany, Section Molecular Developmental Biology,
Faculty of Sciences,
University of Nijmegen,
Nijmegen, The Netherlands

1 Gurdon, J. B., Lane, C. D., Woodland, H. R., and Marbaix, G., *Nature*, 233, 177–182 (1971).
2 Lane, C. D., Marbaix, G., and Gurdon, J B., *J. molec. Biol.*, 61, 73–91 (1971).
3 Moar, V. A., Gurdon, J. B., and Lane, C. D., *J. molec. Biol.*, 61, 93–103 (1971).
4 Gurdon, J B., Woodland, H. R, and Lingrel, J. B., *Devl Biol*, 39, 125–133 (1974).
5 Woodland, H R., Gurdon, J. B., and Lingrel, J. B., *Devl Biol*, 39, 134–140 (1974).
6 Knowland, J., *Genetics*, 78, 383–394 (1974).
7 Donk, J. A. W. M, van der, *Molec. gen. Genet.*, 131, 1–8 (1974).
8 Linskens, H. F., Schrauwen, J. A. M., and Konings, R. N. H, *Planta (Berl.)*, 90 153–162 (1970).
9 Donk, J. A. W. M., van der, *Molec. gen Genet*, 134, 93–98 (1974).
10 Donk, J. A. W. M. van der, *Incompatibility Newslett.*, 4, 30–32 (1974).
11 Laat, S. W., de, Saag, P T. van der, Leurink, W., and Brahma, S. K., *Expl Cell Res*, 84 367–373 (1974).
12 Linskens, H. F., and Esser, K., *Natui wissenschaften*, 44, 16–17 (1957).

Aminoacylation of transfer RNA microinjected into *Xenopus laevis* oocytes

THE microinjection technique perfected by Gurdon and his collaborators[1,2] represents an excellent method for the study of the *in vivo* effects of drastically changing the molecular contents of the injected cells. We have demonstrated[3] that radioactive yeast transfer RNA injected into oocytes of *Xenopus laevis* is not appreciably degraded after 20 h of

incubation inside these cells. It seems therefore, possible to modify the quantity and characteristics of the tRNAs present in frog oocytes and to test the effects that such changes may have on protein synthesis and other cellular processes. An important control in such a study is to determine whether the tRNAs microinjected into frog oocytes are functional in the sense that they can be aminoacylated by the recipient cell. This report presents evidence demonstrating that the amphibian oocyte is capable of aminoacylating yeast tRNAs injected into these cells at concentrations which are greatly in excess of their endogenous tRNA content.

The method used for microinjection of oocytes[3] and for the preparation of protein synthesis elongation factor 1 from wheat embryos[4] have been published previously. Specific
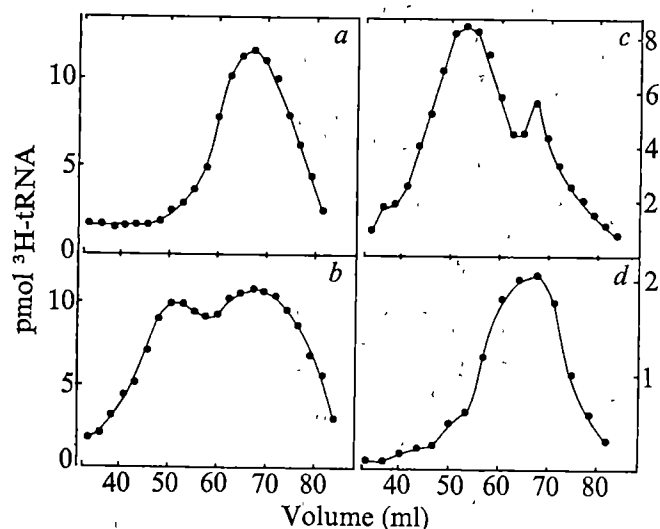
Fig. 1 The aminoacylation of yeast ³H-tRNA injected into *Xenopus* oocytes assayed by the formation of ternary complex with EF1 and GTP. Bulk yeast ³H-tRNA was prepared by the method of Brungraber[12] from a uracil-requiring strain of yeast (13088) grown in ³H-uracil. This method includes a step to deacylate endogenous aminoacyl-tRNAs. Carrier yeast tRNA was added to facilitate isolation. The specific activity of this material was of 500 c.p.m. pmol⁻¹. Electrophoretic analysis showed that this material contained negligible 5S RNA. *a*, 80,000 c.p.m. of the uninjected ³H-tRNA was incubated with 2 mg of wheat EF1 and GTP as described by Tarragó et al.[13] and passed through a Sephadex G-200 column (55 × 1.5 cm) which was equilibrated and eluted with a buffer containing 20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 50 mM NH₄Cl, 1 mM GSH, and 75 μmol GTP. Fractions of 1 ml were collected and aliquots were precipitated with cold 5% TCA, filtered through Millipore membranes and counted. *b*, 54,000 c.p.m. of ³H-tRNA (specific activity 140 c.p.m. pmol⁻¹) was incubated and passed through the same column in identical conditions. This ³H-tRNA has been aminoacylated *in vitro* using a crude wheat embryo aminoacyl-tRNA synthetase solution prepared according to Muench and Berg[14] and a mixture of the 20 natural non-radioactive amino acids. A control experiment showed that the reaction mixture could aminoacylate 1.5% of yeast tRNA with phenylalanine. *c*, Yeast ³H-tRNA recovered after its injection into *Xenopus laevis* oocytes was fractionated in the presence of EF1 and GTP as in *a*. This material containing a total of 120,000 c.p.m. with a specific activity 500 c.p.m. pmol⁻¹, had been injected into 50 *Xenopus laevis* oocytes (50 nl per oocyte) and these cells were incubated at 21 °C for 5 h in amphibian saline. The ³H-tRNA was recovered by phenol extraction as described in Table 1 and the water phase was precipitated with 3 volumes of ethanol at −20 °C. The precipitate was collected by centrifugation at 20,000 g for 30 min and the ³H-tRNA was redissolved in 200 μl of water and added to the ternary complex incubation mixture. *d*, Fractions 40 to 56 of *c* were pooled and precipitated with ethanol as in *c* and the ³H-tRNA was dissolved in 0.5 ml of H₂O and brought to pH 10 with NH₄OH and incubated at 37 °C for 30 min. This treatment deacylates the tRNA and inactivates the wheat EF1 that might be present in these fractions. The mixture was then neutralised with HCl and reprecipitated with ethanol. The precipitate was dissolved in 200 μl of H₂O and incubated with EF1 and GTP as in *a*. 27,000 c.p.m. of ³H-tRNA were run on this column.

details of the experimental procedures are given in the legends to the figures and table.

Controls on the state of the ³H-tRNA recovered after microinjection and on the efficiency of recovery were performed. Electrophoresis on 7.5% polyacrylamide gels according to Peacock and Dingman[5] showed that the material recovered by phenol extraction of oocytes 5 h after microinjection had mobility identical to the original ³H-yeast tRNA. Microinjection of ¹⁴C-Phe-tRNA followed immediately by phenol extraction of the injected cells gave a recovery of approximately 80% of the acid precipitable radioactivity in the water phase.

The small amounts of tRNA that could be injected and recovered from these cells and the necessity to differentiate the injected material from the endogenous tRNA required a new method for the assay of the aminoacylation of total yeast ³H-tRNA. The method of choice determines the capacity of the ³H-tRNA recovered from the injected cells to form a ternary complex with GTP and with protein synthesis elongation factor 1 (EF1) from wheat embryos. It has been demonstrated previously that wheat embryo EF1, like bacterial EF Tu, interacts with aminoacyl-tRNA but not with unacylated tRNA in the presence of GTP[6,7]. Figure 1a shows the elution of uninjected yeast ³H-tRNA on a Sephadex G-200 column after incubation with EF1 and GTP. This preparation which has been chemically deacylated fails to interact with the protein factor and elutes as free tRNA (molecular weight 25,000). In Fig. 1b the same experiment is performed with yeast ³H-tRNA that has been aminoacylated *in vitro* with a crude preparation of wheat aminoacyl-tRNA synthetases and the 20 different non-radioactive amino acids. It is clear that approximately 40% of the tRNA now elutes as a heavier peak (molecular weight 80,000) indicating that by aminoacylation it has acquired the capacity to interact with EF1 and GTP. In a separate experiment in which GTP was absent from the incubation mixture and the column buffer (not shown), the tRNA aminoacylated *in vitro* did not elute with a heavy peak but as in Fig. 1a. Figure 1c shows the results obtained with the original unacylated yeast ³H-tRNA recovered 5 h after its injection into *Xenopus* oocytes and incubated with EF1 and GTP. Its elution pattern has changed drastically from what was observed in Fig. 1a since now 80% of the ³H-tRNA elutes in the ternary complex fraction. To ascertain that this behaviour was due to *in vivo* aminoacylation, fractions 40 to 56 represented in Fig. 1c were pooled and chemically deacylated and, after concentration, run again with EF1 and GTP. Figure 1d shows that deacylation of the tRNA recovered after microinjection destroys its capacity to interact with EF1 and GTP.

It may be concluded therefore that the great majority of the yeast tRNA species microinjected as unfractionated tRNA can be aminoacylated very efficiently by the living oocyte. The amount of yeast tRNA introduced into each cell was approximately 100 ng which is 2.5 times the amount of endogenous tRNA[8].

Direct quantification of *in vivo* aminoacylation in injected oocytes was attained by the use of a pure species of tRNA. The assay consisted in measuring the appearance of radioactivity precipitable with cold TCA in the aqueous phase after phenol extraction of oocytes that had been incubated in a medium containing ¹⁴C-phenylalanine. Table 1 shows that no detectable radioactivity is obtained when uninjected oocytes or oocytes injected with unfractionated tRNA are treated. The amount of tRNA specific for phenylalanine in these cells is too low to be detected in the conditions used. Oocytes microinjected with purified tRNA^Phe, however, show considerable incorporation of radioactivity in the tRNA fraction. The identity of this radioactivity as aminoacyl-tRNA is established by its sensitivity to pancreatic RNase, mild alkaline hydrolysis and to hot TCA treatment. Incubation of the oocytes injected with tRNA^Phe with ¹⁴C-threonine does not yield radioactivity bound to the tRNA. In the table it is also shown that microinjection of tRNA^Phe whose amino acid acceptor capacity

Table 1 The aminoacylation of pure yeast tRNA^{Phe} microinjected into oocytes

| Experiment	Oocytes	Treatment	c.p.m. precipitated with cold TCA per oocyte
1	Uninjected		55
	Injected with 8 pmol of unfractionated yeast tRNA		88
	Injected with 8 pmol of pure yeast tRNA^{Phe}		1,150
	Same as above	^{14}C-Thr instead of ^{14}C-Phe	42
2	Uninjected		38
	Injected with 8 pmol of tRNA^{Phe}		840
	Same as above	RNase hydrolysis	72
	Same as above	pH 10 for 15 min at 37 °C	129
	Same as above	90 °C for 15 min in 5% TCA	49
3	Uninjected		57
	Injected with 8 pmol of pure yeast tRNA^{Phe}		1,285
	Injected with 16 pmol of yeast tRNA^{Phe} oxidised		43

The aminoacylation of tRNA^{Phe} was assayed by incubating duplicate groups of five oocytes each with ^{14}C-phenylalanine of a specific activity 270 μCi μmol^{-1} at a concentration of 3×10^{-5} M for 2 h at 21 °C in 50 μl of a modified Holtfreter's medium containing 20 mM Tris-HCl pH 7.4 and 10 ng ml^{-1} each of penicillin and streptomycin sulphate, 63 mM NaCl, 0.7 mM KCl, 0.9 mM CaCl$_2$, 1.0 mM MgCl$_2$ and 0.22 mM NaHCO$_3$. The oocytes were extracted by homogenising in 4 ml of cold mixture containing 2 ml of buffer (0.2 M sodium acetate 10 mM MgCl$_2$, 5 mM EDTA and 0.1% Triton X-100) and 2 ml phenol. The water phase was separated by centrifugation and duplicate aliquots of 0.5 ml were precipitated with cold 5% TCA. The precipitate was retained on Millipore membranes and the radioactivity was measured in a scintillation counter with 60% efficiency. Unfractionated yeast tRNA was purchased from Calbiochem; pure yeast tRNA^{Phe} from Boehringer. The yeast tRNA^{Phe} was oxidised[11] but the amine treatment not included. When ^{14}C-threonine replaced the radioactive phenylalanine (experiment 1) the specific activity was 181 μCi μmol^{-1} and the concentration of 5×10^{-5} M. In experiment 2, where indicated, 0.5 ml aliquots of the water phase after phenol extraction were treated with 10 μg of pancreatic RNase or with mild alkali or heated in 5% trichloroacetic acid as given in Table 1.

has been destroyed by periodate oxidation fails to incorporate radioactivity.

Figure 2*a* shows the time course of the aminoacylation in oocytes injected with 20 pmol of tRNA^{Phe} per cell. The reaction is not yet complete after 2 h incubation when at least 25% of the injected tRNA has been aminoacylated with ^{14}C-phenylalanine. Exact estimates of the amounts of Phe-tRNA synthesised would have to consider whether the radioactive amino acid is diluted by the internal pool of free amino acid in the oocyte before it is incorporated in the tRNA. The amount of free phenylalanine in *Xenopus* oocytes has been estimated to be approximately 60 pmol per oocyte (Bravo and Allende, unpublished) a quantity that would yield a fourfold dilution of the ^{14}C-phenylalanine taken up by the cell in 2 h. Recent evidence obtained in other cells indicates that this dilution may not take place[9,10].

The effect of injecting different amounts of tRNA^{Phe} on Phe-tRNA formation in the oocytes can be seen in Fig. 2*b*. In this experiment the incubation was carried out for 2 h so that the values obtained with the lower tRNA^{Phe} concentrations represent extent rather than rate of aminoacylation. With limiting amounts of tRNA^{Phe} aminoacylation of 85–90% of the injected tRNA was obtained without considering dilution by the internal pool of free phenylalanine. This result is corroborative evidence that such dilution may not take place in this sytsem.

Using the values shown in these figures it is possible to calculate that each oocyte has enough phenylalanine-tRNA synthetase to catalyse the aminoacylation of approximately
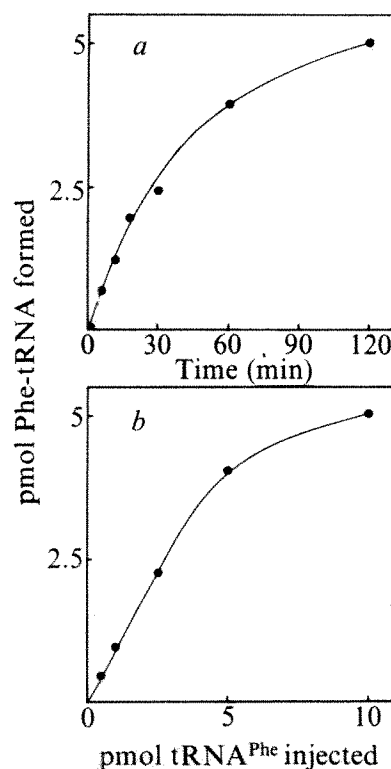
Fig. 2 The effects of time and tRNA concentrations on the *in vivo* aminoacylation of tRNA injected into *Xenopus laevis* oocytes. *a*, 20 pmol of pure yeast tRNA^{Phe} were injected into each oocyte and duplicate groups of five oocytes were incubated at 21 °C for the times indicated in amphibian saline medium containing 3×10^{-5} M ^{14}C-phenylalanine, specific activity of 270 μCi μmol^{-1}. The amount of aminoacylation was determined as described in Table 1 and the pmol of aminoacylated tRNA^{Phe} per oocyte were calculated disregarding cellular dilution of the specific activity of the ^{14}C-phenylalanine. *b*, The amounts of tRNA^{Phe} indicated were injected into each oocyte of duplicate groups of five and incubated for 2 h in the same medium. The amounts of aminoacylation were determined as above.

10^{10} molecules of tRNA^{Phe} min^{-1}. This rate is approximately an order of magnitude higher than the rate of incorporation of phenylalanine into protein in these cells (Bravo and Allende, unpublished).

In conclusion, the results presented above indicate that tRNA microinjected into amphibian oocytes is functional in the sense that it can be aminoacylated *in vivo*. Cells microinjected with tRNA^{Phe} contain more than 500 times the normal concentration of this specific tRNA and 150 times the amount of Phe-tRNA. It should, therefore, be possible in the future to study the effects that such an unbalance provokes on protein synthesis and other cellular processes.

MARTA GATICA
ADELA TARRAGÓ
CATHERINE C. ALLENDE
JORGE E. ALLENDE

Departamento de Bioquimica,
Facultad de Medicina,
Sede Norte Universidad de Chile,
Casilla 6671, Santiago 4—Chile

1 Gurdon, J. B., *J. Embryol. exp. Morph.*, **20**, 401–414 (1968).
2 Gurdon, J. B., Lane, C. D., Woodland, H. R., and Marbaix, G., *Nature*, **233**, 177–182 (1971).
3 Allende, C. C., Allende, J. E., and Firtel, R., *Cell*, **2**, 189–196 (1974).
4 Tarragó, A., Allende, J. E., Redfield, B., and Weissbach, H., *Archs Biochem. Biophys.*, **159**, 353–361 (1973).
5 Peacock, A. C., and Dingman, C. W., *Biochemistry*, **7**, 668–674 (1968).
6 Jerez, C., Sandoval, A., Allende, J. E., Henes, C., and Ofengard, J., *Biochemistry*, **8**, 3006–3014 (1969).
7 Legocki, A., and Marcus, A., *J. biol. Chem.*, **245**, 2814–2818 (1970).
8 Brown, D. D., and Littna, E., *J. molec. Biol.*, **20**, 95–112 (1966).

9 Airhart, J., Vidrich, A., and Khairallah, E. A., *Biochem. J.*, **140**, 539–548 (1974).
10 Van Venrooij, W. J., Moonen, H., and Van Loon-Klaasen, L., *Eur. J. Biochem.*, 297–304 (1974).
11 Baldwin, A. N., and Berg, P., *J. biol. Chem.*, **241**, 839–845 (1966).
12 Brungraber, E. E., *Biochem. biophys. Res. Commun.*, **8**, 1–3 (1962).
13 Tarrago, A., Monasterio, O., and Allende, J. E., *Biochem. biophys. Res. Commun.*, **41**, 765–773 (1970).
14 Muench, K., and Berg, P., in *Procedures in Nucleic Acid Research* (edit. by Cantoni, G. L., and Davies, D. R.), 375–383 (Harper and Row, New York, 1966).

Translation of natural mRNAs *in vitro* by extracts of a mutant in protein synthesis

NATURAL mRNAs contain specific signals for initiation and termination of protein synthesis[1,2]. To further understand these processes and their regulation in the cell, we have been screening mutants defective in translation of natural mRNAs but not of artificial templates.

The temperature-sensitive *Escherichia coli* mutant, N4316, is one of this class. At 36 °C, *in vivo*, N4316 suppresses the termination codons UAA and UGA, but not UAG (ref. 3). At
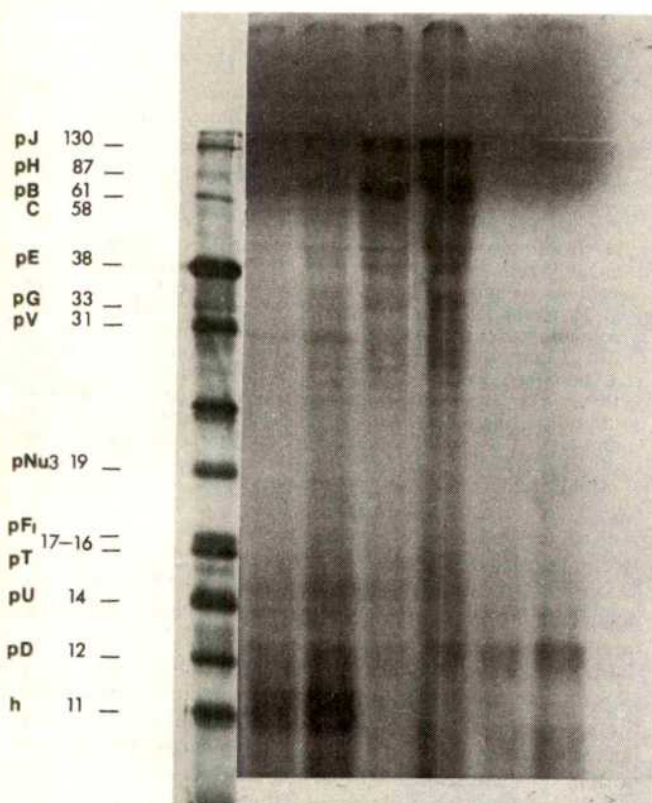
Fig. 1 Analysis of MS2 and MS2 (623) products on SDS polyacrylamide gels was according to Maizel[16] using 15 % acrylamide and 0.8 % bisacrylamide in the separating gel and 5 % acrylamide in the stacking gel. Products were labelled with ^{35}S-methionine (see Table 2). Incubations were terminated with 13 mM EDTA, and 2 μg RNase I. After 8 min at 35 °C, proteins were concentrated by precipitation with 5 volumes of acetone at −20 °C. Electrophoresis was at 90 V for 1 h and 180 V until the tracking dye reached the end of the gels. X-ray films (RP X-Omat, Kodak Corp) were exposed for 4 d. Bacteriophage λ proteins labelled with ^{35}S-methionine were a gift from C. Epp and P. Ray (University of Toronto). Samples from left to right were as follows: λ proteins, 200×10^3 c.p.m.; MS2, 20.4×10^3 c.p.m.; MS2, 40.8×10^3 c.p.m.; MS2 (623), 28.0×10^3 c.p.m.; MS2 (623), 56.0×10^3 c.p.m.; no RNA, 9.4×10^3 c.p.m.; no RNA, 18.8×10^3 c.p.m. Counting efficiency for ^{35}S was 50 %. The leftmost channel contains phage λ late polypeptides labelled *in vivo* with ^{35}S-methionine. These are indexed according to their gene of origin; 'h' is a host peptide(s). The numbers refer to the molecular weights of these peptides in kdaltons. By extrapolation 'h' has a minimum molecular weight of 10,500. The major product of *in vitro* synthesis (coat protein) migrates like a peptide of molecular weight 10,800±300. ^{125}I-labelled (or unlabelled) authentic coat protein[11] was found repeatedly to have the identical migration in these gels as the *in vitro* synthesised product.

Table 1 Protein synthesis by N4316 extracts programmed with amber mutants of the maturation and replicase cistrons of MS2 RNA

RNA	d.p.m. ^{3}H-histidine incorporated ($\times 10^{-3}$)		
	36 °C	46 °C	46 °C+RF
MS2	194	38.4	146
*am*5	41.0	23.5	66.0
*am*9	152	35.0	113

All experimental details were as described previously[7] except that incubations contained 5 μCi ^{3}H-histidine (58 Ci mmol^{−1}, Radiochemical Centre). Where indicated, 1.3 μg of rescue factor (RF) prepared without the DEAE cellulose step[7] and 12.5 μg of each mRNA were used to programme the S30 extracts of N4316. Radioactivity incorporated into hot TCA-insoluble products was measured. *Am*5 and *am*9 lysates, gifts of Dr M. Kozak and D. Nathans (Johns Hopkins University) were grown as described[9], and contained less than 0.1 % revertants. Controls with extracts from the parental strain D₁₀, showed no temperature sensitivity and no stimulation by the rescue factor. Incorporation without mRNA, about 10 % of the total, was subtracted from each value. Counting efficiency for ^{3}H was 20 %. Products were analysed as described by Ganoza *et al.*[5]. The bulk of the released MS2 and *am*9 products correspond in molecular weight to maturation protein (40,000).

30 °C the mutant does not suppress[3]. This observation implied a temperature-dependent lesion in termination, and a moderate defect in this function was demonstrated *in vitro*[4–6]. The mutation, however, seems pleiotropic. At 43–45 °C, translation *in vivo* is impaired and cells die[3]. In extracts of N4316 synthesis of the coat protein coded by R17 and f2 phage RNAs is greatly reduced at restrictive temperatures[4–6]. The defect *in vitro* seems to be in an early function of translation[7]. This function does not involve the initiation reaction in the usual sense, since the rate and extent of formation of the Fmet-tRNA–ribosome–f2 RNA complex is unimpaired[6,7]. The synthetic defect can be reversed by adding a soluble protein from wild type cells[7]. This "rescue" protein has been purified and is free of known enzymes and factors involved in protein synthesis[7].

In this communication we further examine the temperature-sensitive defect in *in vitro* synthesis and its reversal by measuring systematically the reading of a number of cistrons from a spectrum of phage mRNAs. The measure of translation of specific cistrons was made possible through the use of mRNAs bearing known amber mutations. Our data indicate that the early translation lesion is general, and is the more drastic and important defect. Furthermore, the early defect in cistron reading is not the result of an interference with initiation by termination intermediates in the intragenic regions of the mRNAs.

To score for specific cistrons, histidine was used as label because it is not present in the coat protein and synthesis was programmed by early amber mutants of the maturation cistron (*am*5) or the replicase cistron (*am*9), so that only one protein was measured at a time. Protein synthesis in extracts of N4316 directed by *am*9 is temperature sensitive and can be restored by rescue factor (Table 1). The *am*9 mRNA directs synthesis and release of a product that corresponds in molecular weight to the maturation protein (unpublished).

Protein synthesis catalysed by N4316 extracts using the *am*5 template is also temperature sensitive and the defect is reversed by rescue factor (Table 1). Analysis of the products on SDS polyacrylamide gels done after separation of nascent and free proteins showed that the smaller level of translation with *am*5 at 36 °C is due to a low level of replicase synthesis (unpublished). To overcome this problem, an amber mutant of the coat protein which overproduces replicase was used. As shown in Fig. 1, MS2 (623) RNA, programmes a product corresponding in molecular weight to the phage replicase. The synthesis of this product is temperature sensitive in N4316 and is reversed by the rescue factor (Table 2, experiment 1). Protein synthesis directed by Qβ RNA is also temperature sensitive and is restored by the rescue protein (Table 2, experiment 5).

These data show that the rescue protein works on all phage

cistrons and with all mRNAs tested (R17, MS2, f2 and Qβ). The maturation protein is coded by the 5'-terminal segment of the phage RNA. Since synthesis of this protein is defective in extracts of N4316 (Table 1), it is unlikely that the impaired synthesis at restrictive temperatures is the result of a defect in the release mechanism.

Further evidence that the defect cannot be accounted for by a problem in termination only is given in Table 2. Synthesis programmed with a non-polar mutant (*am*11b) of R17 phage is thermolabile and is reversed by the rescue protein. In *am*11b, the UAG codon is 59 codons away from the normal signal for termination of coat protein synthesis. Synthesis programmed by *am*11 of f2 is also temperature sensitive (Table 2). This phage has two amber mutations, one in the maturation cistron, and the other at the 50th codon of the coat protein cistron. These experiments indicate that steric hindrance by ribosomes is not responsible for the impaired translation.

No coat protein is produced when the MS2 (623) RNA is used as messenger (Fig. 1). This rules out misreading of the amber codons by the N4316 strain. These experiments show that the mutant N4316 has a marked effect on translation

Table 2 Protein synthesis in extracts directed by different mRNAs

Experiment no.	RNA	d.p.m. incorporated ($\times 10^{-3}$)			
		40–41 °C		43–46 °C	
		−RF	+RF	−RF	+RF
1	MS2 (623)	229	259	62.0	110
2	R17	327	—	12.5	195
	*am*11b	254	—	19.0	206
3	R17	181	—	18.5	152
	*am*11b	217	—	16.5	176
4	f2	368	415	65.6	201
	*am*11	329	328	50.4	197
5	Qβ	761	—	35.5	345

Experimental details were as described (ref. 7 and Table 1) except that 5 μCi of ^{3}H-lysine (3.7 Ci mmol^{-1}, New England Nuclear) as used in experiments 1, 2 and 5; 5 μCi ^{35}S-methionine (147 Ci mmol^{-1}, New England Nuclear) in experiment 4; and 5 μCi ^{3}H-histidine in experiment 3. Where indicated 2.3 μg of RF were used. Phages MS2 (623) (a gift from Gordon Carmichael, Harvard Biolabs) and Qβ (a gift from David Hoar, University of Toronto) were grown and purified and the RNA extracted as described previously[9]. Revertants were less than 0.1%. Where shown, synthesis was programmed by 11 μg of each RNA. Radioactivity incorporated into acid-insoluble products was measured. Optimum temperatures for synthesis were used as permissive temperatures as follows: experiments 1 and 4, 40 °C; and experiments 2, 3 and 5, 41 °C. Non-permissive temperatures were as follows: experiment 1, 43 °C; experiments 2, 3 and 4, 45 °C, and experiment 5, 46 °C. S30 extracts from N4316 were used in these experiments. Controls with the parental strain, D$_{10}$, showed no temperature sensitivity, and no stimulation by rescue factor with any of the RNAs. The predominant ^{35}S-labelled products directed by R17, f2 or Qβ RNAs at 45 or 40 °C, with or without rescue factor, migrated on 10% acrylamide gels (ref. 10 and Fig. 1), with identical mobility to authentic coat protein extracted from f2 phage. Products smaller than coat protein were observed with MS2(623) or *am*11 RNA. Other bands observed after prolonged exposure with Qβ or f2 RNA with and without factor were about 65,000 and 35,000–40,000 in molecular weight.

apart from its effect on chain termination. This defect affects translation of all mRNAs tested and all cistrons examined to approximately the same extent.

Other mutants of the same class exhibit similar phenotypes (unpublished). We therefore suggest that there exist functions vital to translation or its regulation in the cell which are not scored by any of the known partial assays of translation.

We thank Miss A. Tırpak for help in growing phages and Dr J. P. van der Meer for a gift of rescue factor used at the beginning of this work. This work was supported by an MRC of Canada grant to M.C.G.

M. B. HERRINGTON
M. J. DOHERTY
M. C. GANOZA

Banting and Best Department of Medical Research,
University of Toronto,
Toronto, Ontario, Canada

1 Ochoa, S., and Mazumder, R., *The Enzymes*, third ed., 10 (edit h · Boyer, P. D.), 1–86 (Academic, New York and London, 1974)
2 Tate, W. P., and Caskey, T. C., *ibid*, 87–118.
3 Phillips, S. L., Schlessinger, D., and Apirion, D., *Cold Spring Harb. Symp quant. Biol*, 34, 499–503 (1969).
4 Ganoza, M. C., van der Meer, J., Debrecent, N., and Phillips, S. L., *Proc. natn. Acad. Sci. U S.A.*, 70, 31–35 (1973).
5 Ganoza, M. C., and Phillips, S L., *Fedn Proc*. 30, 1382 (1971).
6 Phillips, S. L., *J. molec. Biol.*, 59, 461–472 (1971).
7 Van der Meer, J., and Ganoza, M. C., *Eur. J. Biochem.*, 54, 229–237 (1975).
8 Kozak, M., and Nathans, D., *Bact. Rev.*, 36, 109–134 (1972).
9 Webster, R E., and Zinder, N. 1', *J. molec. Biol.*, 42, 425–439 (1969).
10 Maizel, J. V., Jr, in *Methods in Virology*, 5 (edit. by Maramorosch, K, and Koprowski, H), 179–249 (Academic, New York, 1969).
11 Hunter, W. M, and Greenwood, F. C, *Nature*, 194, 495–496 (1962).

Symmetry recognition hypothesis model for tRNA binding to aminoacyl-tRNA synthetase

THE fidelity of the transfer of the genetic information at the translational level depends largely on the accuracy with which each transfer RNA (tRNA) is aminoacylated by its cognate synthetase. The search for the common sites on tRNA which the enzyme recognises has resulted so far in a rather confusing picture. Practically all parts of the tRNA molecule have been implicated as the possible recognition site(s)[1,2].

Based on the general three-dimensional structure of tRNA (ref. 3) and the observation of the repeating sequences in various aminoacyl tRNA synthetases[4-7], a model for the binding of tRNA and aminoacyl tRNA synthetase (aa-RS) is proposed where the internal symmetry of tRNA coincides with that between "unit domains" of the enzyme.

The three-dimensional structure of two crystal forms of yeast phenylalanine tRNA has been determined by the X-ray crystallographic method[8-10]. The structure is shown in Fig. 1. An examination of the structure reveals that the molecule contains two types of symmetry elements relating two regions: a pseudo twofold axis and a pseudo twofold screw axis within a molecule as shown in Fig. 2. The regions related by the twofold axis overlap with, but are larger than, those related by the screw axis.

Some aa-RSs are composed of multiple subunits of the "smaller protomer" (molecular weight 35,000–50,000) and others contain one or more subunits of the "large protomer" (molecular weight 70,000–100,000)[1,2]. It has been suggested that the "large protomer" is composed of two homologous sequences[4-7]. This sequence homology implies that the three-dimensional structure of the protomer is likely to contain two structural domains as was the case in the crystal structure of Fab' fragment of a human immunoglobulin[11], where the two structural domains in one chain are related to each other by a pseudo screw axis. A preliminary X-ray crystallographic study of *Escherichia coli* methionine tRNA synthetase, part of which was enzymatically cleaved off, shows a structural domain of a prolate ellipsoidal shape (Reisler, J.-L., unpublished) with approximate axial lengths $22 \times 22 \times 30$ Å. The estimated molecular weight corresponding to the volume of this structural domain (based on the assumption that the partial specific volume of the average protein is 0.74 ml g^{-1} (ref. 12)), 49,000, which is in the range of the expected value of 43,000 for one half of the protomer. The most likely symmetry elements relating two structural domains in such a protomer are a pseudo twofold axis and a pseudo screw axis. Thus, one such structural domain can be considered a "unit domain" and two or more such "unit domains", which are related by pseudo twofold and/or screw axes, could form the assumed three-dimensional structure of a functional enzyme.

The model proposed here is based on the following assumptions: First, the aa-RSs recognise the common tertiary structural features of tRNAs. This is supported by the observations that the misacylation occurs in heterologous systems. (for example, *E. coli* tRNAs charged by yeast enzymes[13,14]) as well as in homologous systems[14]. Second, the specific recognition then selects the cognate mates. The hypothesis put forward is that the pseudo-symmetric regions of a tRNA are recognised

by the pseudo-symmetric regions of an aa-RS consisting of more than one "unit domain". For the enzymes with two identical small protomers, the true symmetry is assumed to convert to the pseudo symmetry by an asymmetric conformational change either on dimerisation or on ligand binding. This "symmetry recognition" can be achieved by coinciding the symmetry axes of the same type from tRNA and the enzyme, for example, the pseudo twofold axis of the tRNA with the pseudo twofold axis of the enzyme, while maximising the contact area as shown schematically in Fig. 3. In making the schematic complex, the regions of nucleotide sequence which vary the most among different tRNAs (dotted portion in Fig. 1) are kept further away from the enzyme and the overall contact area on tRNA is localised more on the "inside or diagonal side" of the L-shaped tRNA as has also been suggested by Rich and Schimmel (unpublished).

The symmetry-matching process will align tRNA and the enzyme in register and provide contact areas including some
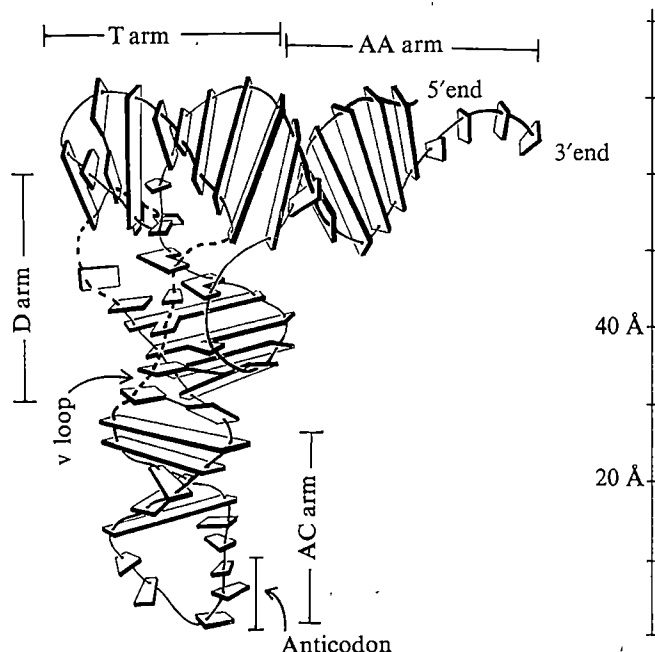
Fig. 1 Schematic drawing of the 3-D structure of yeast phenylalanine tRNA in the orthorhombic crystal[9]. The amino acid acceptor (AA) arm and the TψC (T) arm form a continuous double helix and the anticodon (AC) arm and the DHU (D) arm form the other partially continuous double helix. These two helical columns form an "L", where the D and T loops are at the corner of the "L". The phosphate–ribose backbone is shown as a continuous wire except for the three dotted segments, variable (V) loop and two segments in the D loop. The number of nucleotides in these three segments varies among different tRNAs. The secondary and tertiary base pairs are shown as long or bent slabs and the additional tertiary hydrogen bonds between the bases are shown as dark straight lines. Tertiary hydrogen bonds involving riboses and phosphates are not shown. The molecule is rather flat with the approximate thickness of 20 Å. The backbone conformation at the 3'-end in the crystal is more extended than shown here.

non-symmetric regions. The contact areas on the enzyme are not expected to be one continuous elongated crevice, but two partially isolated regions, one on each "unit domain". Similarly, the contact areas on tRNA are expected to be centred around parts of two symmetric regions and their neighbourhoods. There are two alternative general contact areas on tRNA as shown in Fig. 3c and d, both of which include two symmetry-related regions (AA and AC stems) as well as parts of the AC loop and the D stem (for abbreviations, see the legend of Fig. 1). Most of the implicated enzyme recognition sites (AA stem[1,2,15], AC stem[16], D stem[13], anticodon[1,2,17]) belong in the symmetric regions or their neighbourhoods. More of these implicated recognition sites are in contact with the enzyme in the model shown in Fig. 3c than the one in 3d.
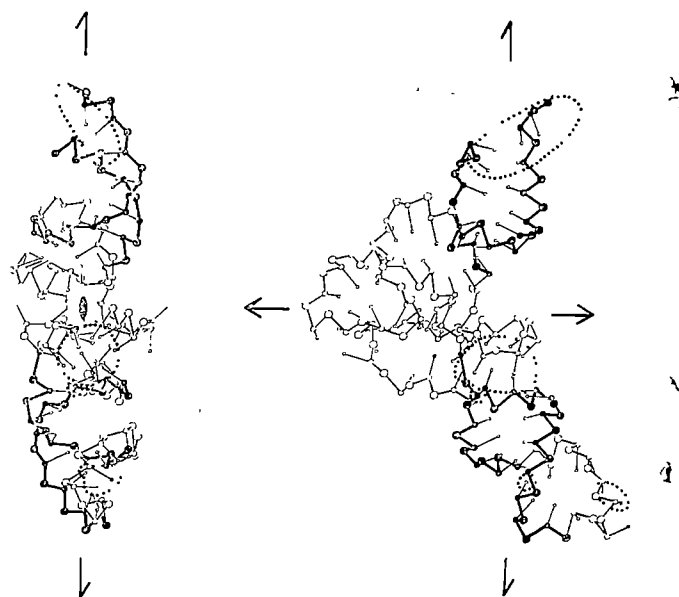
Fig. 2 Two views of yeast phenylalanine tRNA in the orthorhombic crystal[9]. The large, medium and small circles represent the centres of the phosphates, the riboses, and the bases, respectively. The pseudo twofold axes are indicated by arrows when on the plane and by a solid ellipse when perpendicular to the page. The pseudo twofold screw axes are indicated by one-headed arrows. The twofold symmetry related backbones are shown in black. The backbones related by twofold screw axes are shorter than those related by twofold axes. A few nucleotides at the 3'-terminal end are not shown. The aa-RS recognition sites implicated by various experiments[1,2,13,15-17] are circled by dotted lines.

The specific recognition sites of tRNA for the cognate enzymes are likely to be different for different tRNAs within the contact areas, but the common scheme by which tRNA and the enzyme align is proposed to be by the recognition of the pseudo-symmetric regions on both molecules.

The model proposed here enables one to make a few predictions. First, any increase or decrease in the symmetry in the pseudo symmetric regions (AA and AC stems primarily) will lower tRNA-enzyme binding properties. Such change of the symmetry can occur when a base pair is replaced by an unusual base pair such as a GU pair, or vice versa. The charging specificity may or may not change because the specific recognition site(s) may be in or in the neighbourhood of the symmetric regions. Second, any alterations in the T arm or D loop (and V loop in case of the model shown in Fig. 3c) which do not change the relative positions of the AA and AC stems and the pseudo-symmetry between them will not affect the binding or charging.

Fig. 3 Schematic drawings showing the matching of the pseudo twofold symmetry axes of tRNA and aa-RS: two orthogonal views of a, tRNA; b, two symmetry related "unit domains" in an aa-RS; and c and d, two alternative ways of forming tRNA-enzyme complex models by coinciding the twofold axes of both molecules. The matching of the pseudo screw axes can be achieved in a similar fashion with slightly different arrangement between the two molecules.
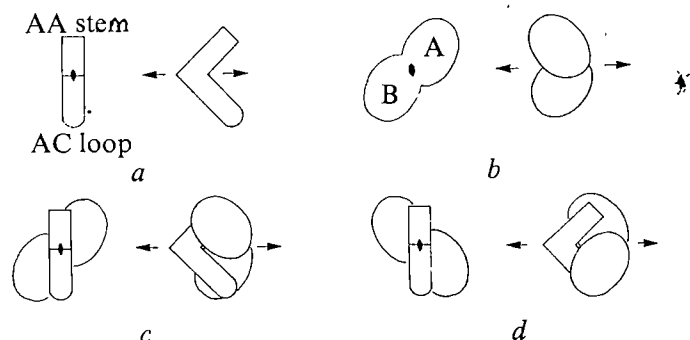
specificity. Third, for each model complex (Fig. 3c or d) there are two ways tRNA can interact with the enzyme: one is as in Fig. 3c or d, the other is by keeping two "unit domains" the same but rotating tRNA by 180° around the pseudo twofold axis, allowing the AC stem to interact with "unit domain" A and the AA stem to interact with B (see Fig. 3b). Since the symmetry is not true, these two modes of binding are likely to show two different tRNA binding properties.

This research was supported by grants from the National Institutes of Health and the National Science Foundation. I thank Alex Rich and Paul Schimmel for a preprint describing their model, Jean-Loup Risler for the unpublished results of the X-ray studies on modified *E. coli* methionyl-tRNA synthetase, and Joel Sussman and Marilyn Warrant for assistance. *Note added in proof:* The same hypothesis can be applied to the recognition between tRNA and other proteins with more than one domain or subunit, such as reverse transcriptase and its primer tRNA.

SUNG-HOU KIM

Department of Biochemistry,
Duke University Medical Center,
Durham, North Carolina 27710

1 Kisselev, L L., and Favorova, O. O., *Adv. Enzymol.*, **40**, 141 (1974).
2 Soll, D., and Schimmel, P. R., *Enzymes*, **10**, 489 (1974).
3 Kim, S. H., *et al.*, *Proc. natn. Acad. Sci. U S A.*, **71**, 4970 (1974).
4 Kula, M.-R., *FEBS Lett.*, **35**, 299 (1973).
5 Koch, G. L. E , Boulanger, Y., and Hartley, B. S , *Nature*, **249**, 316 (1974).
6 Waterson, R. M., and Konigsberg, W. H , *Proc natn Acad. Sci. U.S.A.*, **71**, 376 (1974)
7 Bruton, C. J., Jakes, R., and Koch, G. L. E., *FEBS Lett.*, **45**, 26 (1974).
8 Kim, S. H., *et al.*, *Science*, **179**, 285 (1973).
9 Kim, S. H., *Science*, **185**, 435 (1974).
10 Robertus, J. D , *et al*, *Nature*, **250**, 546 (1974).
11 Poljak, R. J., *et al.*, *Proc. natn. Acad. Sci U S.A.*, **70**, 3305 (1973).
12 Mathews, B W., *J molec Biol.*, **33**, 491 (1968).
13 Roe, B., Sirover, M , and Dudock, B., *Biochemistry*, **12**, 4146 (1973).
14 Giegé, R., *et al.*, *Eur. J. Biochem.*, **45**, 351 (1974).
15 Ghysen, A., and Cellis, J. E., *J. molec. Biol.*, **83**, 333 (1974).
16 Anderson, K W., and Smith, J. D , *J. molec. Biol.*, **69**, 349 (1972)
17 Yaniv, M., Folk, W. R., and Berg, P , *J. molec. Biol.*, **86**, 245 (1974).

Influence of conformation on effect of macroions on kinetics of ionic reactions

POLYELECTROLYTES are known to alter drastically the rate of many ionic reactions in solution[1,2]. In general, it may be said that the polyelectrolyte effect has an important role in the observed changes of the rates of reaction. The high electrostatic field in the neighbourhood of the macroion generates an inhomogeneous distribution of the ionic reactants—counterions being concentrated in a rather small volume surrounding the polymeric chains. It has been clearly shown[3,4] that even when the ionic substrate is decomposed by specifically reactive groups incorporated into the polyelectrolyte molecule, the electrostatic interaction is the causative factor of the observed accelerated rate of decomposition. This polyelectrolyte effect may be a relevant factor on the activity of catalytic bio-polyelectrolytes, for example enzymes.

So far, almost all the polymers studied have had a random coil conformation. Since conformation is a very important parameter in determining the catalytic activity of natural biopolyelectrolytes, we compare here the accelerating power of the α-helix conformer of a poly(amino acid) in solution to that observed in the presence of the corresponding random coil conformer.

Previously, the effect of the conformation of poly(L-glutamic acid) in solutions on the rate of polymerisation of vinylpyridine has been reported[5]. Rates were found higher for the α-helix conformer, but the mechanism and interpretation of the effect is not straightforward. Overberger and Podsiadly[6] have demonstrated the importance of polymeric chain aggregation on the rate of a solvolytic reaction. This conformation involves only intermolecular hydrogen bonding, consequently it has no bearing on intramolecular hydrogen bonding occurring in biopolymers.

The rate of decomposition of 2,4-dinitrophenylphosphate
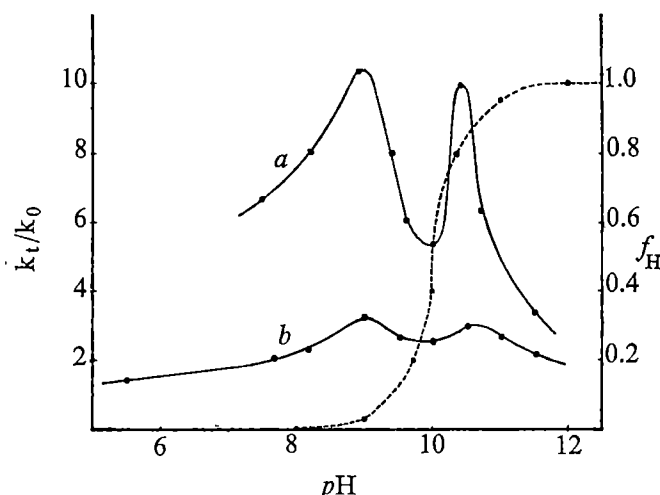
Fig. 1 Acceleration factor k_t/k_0 ($k_0 = 0.19 \times 10^{-4}$ s^{-1}) for the decomposition of DNPP against pH in the presence of poly (L-lysine). a, $R = $ [poly(L-lysine)]/[DNPP] $= 290$; b, $R = 100$. Dashed line, fraction of α-helix, f_H, of poly(L-lysine) against pH (refs 7 and 10). ●, present work. The substrate decomposition was followed spectrophotometrically at 358 nm. At this wavelength the two reaction products, dinitrophenol and dinitro-(substituted aniline), absorb in alkaline solutions. Alternatively, the formation of the aniline alone may be followed in acidic medium ($pH < 3$) at the same wavelength[3,8,9]. All the rate measurements were performed at 30 ± 0.1 °C, and the course of the reaction followed up to 50% conversion; in this range the kinetic law is strictly first order for DNPP.

(DNPP) in solutions of poly(L-lysine) (PLL), a poly(amino acid) capable of undergoing a pH-sensitive α helix $\rightleftharpoons$ random coil conformational transition[7], was determined. DNPP reacts specifically with the free-NH$_2$ groups in simple amine solutions[8,9] as well as in poly(ethyleneimine) solutions (E.B., R.F.-P., and D.T., unpublished), in an analogous fashion to p-nitro-phenyl phosphate[3]. The same reaction takes place with the free-NH$_2$ groups in PLL. The DNPP decomposition occurs by way of the dianion ($pK_2 = 4.5$) throughout all the pH range studied. In this pH region the hydrolysis of DNPP in water is independent of pH (refs 8 and 9 and E.B., R.F.-P., and D.T., unpublished).

The conformational transition of PLL in the presence and absence of the substrate was followed with circular dichroism measurements (JASCO J-20 Recording Spectropolarimeter). As shown in Fig. 1, the transition is not affected by the presence of the substrate and the values agree with those reported in the literature[7,10].

Figure 1 shows the results of the kinetic experiments at various pH. The acceleration of the decomposition is expressed as k_t/k_0, k_t being the first-order rate constant in poly(amino acid) solutions at each pH value. The rate constant k_0 is the corresponding value in water since at the concentrations used in this work simple amines do not contribute significantly to the rate of decomposition of DNPP[3,8,9]. Figure 2 shows the pH profile of k_t/k_0 for DNPP decomposition in solutions of poly(DL-lysine) (PDLL), which does not undergo the α helix $\rightleftharpoons$ coil transition; at all pH values it exists as a random coil[11]. The maximum at pH 8.9 in PLL solutions (Fig. 1) and that observed in PDLL solutions (Fig. 2) correspond to the effect of the random coil polyions on the rate of DNPP decomposition. A single maximum has also been observed for the decomposition of this substrate and p-nitrophenyl phosphate in solutions of random coil macroions possessing -NH$_2$ groups in their molecules (ref. 3 and E.B., R.F.-P., and D.T., unpublished). At the pH of the first maximum in Fig. 1, PLL is 98% random coil. These maxima are accounted for by the decreasing charge density of the weak polyelectrolyte, and by the increasing number of free -NH$_2$ groups when the pH of the solution increases (which may be considered similar to bifunctional catalysis). Further support for this interpretation

is given by the data illustrated in Fig. 3. It may be seen that k_t/k_0 for PLL random coil increases up to a constant maximum value as the concentration ratio R = [PLL]/[substrate] increases. The constant k_t/k_0 value is attained when all the dianion is concentrated close to the chain where the reaction with $-NH_2$ groups takes place. The minimum value of R at which k_t/k_0 becomes constant is dependent on the charge of the substrate and on the charge density on the macroion, as demonstrated in previous work[3,4].

The model postulates two stages for the DNPP reaction: first, attraction of the substrate dianion to the vicinity of the polymeric chain, and second, attack of DNPP ions by the free–NH_2 groups on the macroion. Any change of charge density or number of free amino groups must give rise to changes in k_t/k_0. One way of producing these changes is to alter the pH, as shown in Fig. 2 and the first maximum in Fig. 1. Another way is to alter the conformation of PLL, the transition taking place at pH 10.0. Figure 1 shows that a second maximum appears at pH 10.4 in the α-helix region (82% of PLL is in the α-helix conformation at pH 10.4). In spite of the smaller degree of ionisation of PLL in the alkaline solutions, where the second maximum is observed, the fact that the average distance between adjacent lateral groups, measured along the polymeric chain, is reduced from 3.6 Å, for the random coil, to 1.5 Å for the α helix[11], produces a marked increase in k_t/k_0. Thus, a new maximum appears in the accelerating factor due to the sudden increase in charge density of PLL and possibly also, in the availability (or increase in local concentration) of $-NH_2$ groups in the α-helix conformer. The magnitude of the factor by which the α helix is more effective than the random coil in accelerating the rate of DNPP decomposition was estimated using the equation,

$$(k_{helix}-k_0)/(k_{coil}-k_0)$$

The rate constants k_{helix} and k_{coil} for the pure conformers were calculated from the observed k_t and the fraction of α helix (f_H). The comparison was made with data corresponding to pH 10.4, the α helix being 5.5 times more effective than the random coil. If the data refer to a degree of ionisation of 0.11 for the amino groups of both conformers, the α helix becomes 12 times more effective.

The proposed model is so far only qualitative and it is

Fig. 3 k_t/k_0 against R, measured at degree of neutralisation, $i = 0.9$. ●, [DNPP] = 6.7×10^{-6} M, plus the corresponding amounts of PLL; ×, [DNPP] = 8.3×10^{-6} M, [PLL] = 2.4×10^{-3} N; ○, [DNPP] = 2.4×10^{-5} M, [PLL] = 2.4×10^{-3} N; □, [DNPP] = 8.3×10^{-6} M, [PLDL] = 3.3×10^{-3} N.

probable that other interactions (hydrogen bonding, local environment and so on) also contribute to some extent.

M. A. Blesa
D. Turyn

Department of Physical Chemistry,
Facultad de Farmacia y Bioquímica,
Universidad de Buenos Aires

R. Fernandez-Prini*

Sector Electroquímica Aplicada,
Instituto Nacional de Tecnología Industrial,
Buenos Aires

E. Baumgartner†

Area Investigación y Desarrollo,
Departamento de Química,
Comisión Nacional de Energía Atómica,
Buenos Aires, Argentina

*To whom correspondence should be addressed.
†Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

1 Morawetz, H., *Accs Chem. Res*, 3, 354–60 (1970).
2 Ise, N., *Adv. Polymer Sci*, 7, 536–93 (1971).
3 Fernández-Prini, R, and Turyn, D., *J Chem. Soc. Faraday I.*, 69, 1326–37 (1973).
4 Fernández-Prini, R., and Baumgartner, E, *J. Am. Chem. Soc.*, 96, 4489–94 (1974).
5 Kabanov, V., Ptrovskaya, V., and Kargin, V., *Vysokomolekul Soedin*, A10, 925–31 (1968)
6 Overberger, C. G., and Podsiadly, C. J., *Bioorg. Chem.*, 3, 16–34 (1974).
7 Applequist, J, and Doty, P., in *Polyamino Acids, Polypeptides and Proteins*, (edit. by Stahmann, M. A), 161–77 (University of Wisconsin Press, Wisconsin, 1962).
8 Kirby, A. J, and Varvoglis, A. G., *J. Am Chem Soc*, 89, 415–23 (1967), *J. Chem. Soc*, (*B*), 135–41 (1968).
9 Kirby, A. J., and Jencks, W P., *J Am. Chem. Soc.*, 87, 3209–16, 3217–24 (1965).
10 Townend, R., Kumosinski, T., Timasheff, S, Fasman, G, and Davidson, B, *Biochem. biophys. Res. Commun.*, 23, 163–9 (1966).
11 Lehninger, A L, *Biochemistry*, ch. 6 (Worth, New York, 1970).

Fig. 2 k_t/k_0 against pH for the decomposition of DNPP in the presence of poly(DL-lysine) (R = 400).

Errata

reviews

THE current generation of physicists and engineers is probably more familiar than any of its predecessors with the name of Fourier because of the many modern applications of Fourier analysis and Fourier transforms, which range from diffraction theory and spectroscopy to circuit analysis and quantum mechanics. In the 19th century Fourier profoundly influenced Kelvin and Heaviside; and as early as 1833 William Rowan Hamilton was writing of "Fourier, whom I place at the head of the French School of Mathematical Philosophy, even above Lagrange and Laplace".

So the prospective reader of a biography of Fourier* might expect to find an account of a typically great theorist, probably withdrawn from the buffetings of normal life, in accordance with the popular picture of a man of science. But that picture is open to question, especially as regards those generations of French scientists of which Fourier was a member. Maupertuis fought in the army of Frederick the Great. Lazare Carnot was the "organiser of victory" and the planner of Napoleon's most successful campaign. Charles, of Charles' Law, was one of the crew of two in the first manned flight of a hydrogen balloon. And Gay Lussac for many years held the world's balloon altitude record of 23,000 feet, which he established in 1804. But as is well brought out in Dr Herivel's new biography, none of these experiences was more intense than those of Fourier, whose life combined science and action in a remarkable way. When he started his classical work on the theory of thermal conduction he was about 35 years old—a distinctly late beginning

Joseph Fourier: The Man and the Physicist. By John Herivel. Pp. xi+350 +5 plates. (Oxford University Press: London; Clarendon: Oxford, April 1975.) £9.75.

Physicist extraordinaire

R. V. Jones

for a theorist—and he had already had a most adventurous career.

Born at Auxerre in 1768, he went to the Ecole Militaire in that town, and thence to the Abbey at St. Benoit with the intention of entering the Benedictine Order. Returning to Auxerre without taking his vows, he was made an Abbé in 1790; but he became so caught up in the maelstrom of the French Revolution as to be named at one time for execution; and a little later he became President of the Revolutionary Committee in Auxerre and, as such, a chief agent of the Terror. As so often happened, this republican prominence rebounded, and he himself was imprisoned in 1794. Released, he was nominated to the Ecole Normale, only to be imprisoned once more in Paris in 1795. Again released he went to the École Polytechnique, where in 1797 he succeeded Lagrange in one of the Chairs. In 1798 he was summoned to join Napoleon's Expedition to Egypt, along with many other eminent men of learning. After the military defeat of

this Expedition, Fourier was held as hostage for all the other savants by Admiral Sir Sidney Smith, an experience which resulted in friendship rather than resentment, for the Admiral saved Fourier's papers from destruction and later returned them to him in France.

Fourier became Prefect of the Department of Isère and was stationed at Grenoble, where he succeeded in organising the draining of the swamps of Bourgoin, a project which had been afoot since the time of Louis XIV. At the same time he was editing the general introduction to the *Description of Egypt*, the great compilation of the results of the Egyptian Expedition. Created a Baron in 1802, he was still Prefect of Isère in 1815— and still at Grenoble, right in the path of Napoleon's famous march on Paris. Vacillating about the surrender of Grenoble to his old Emperor, who had to batter the gates open after Fourier had conveniently withdrawn himself to the country, he incurred Napoleon's displeasure and was dismised from his post as Prefect, only to be appointed Prefect of the Rhône by a mollified Napoleon a few days later. But after Waterloo, Fourier's Napoleonic favours did not endear him to Louis XVIII, and so within three months he was removed from his second prefecture. Louis indeed refused for a time to confirm Fourier's election to the Academie des Sciences but relented in 1817, and from 1822 until his death in 1830 Fourier was permanent Secretary of the Academie. All these events are described with great historical care by Dr Herivel, who has provided an illuminating selection of Fourier's letters, along with many helpful biographical notes about the multitude of characters with whom Fourier became involved.

It was during his period in Grenoble that Fourier worked on the conduction of heat; and, as Dr Herivel emphasises, his first basic contribution was to

postulate that the flow of heat could be treated as that of an indestructable substance. In view of its antecedence in the caloric theory, and of its subsequent universal acceptance, it is hard to appreciate that Fourier had great difficulty in persuading Laplace, Biot and Poisson of its validity. Their doubts about his physics were reinforced by parallel doubts about his non-rigorous treatment of trigonometrical series, but in both instances his instincts proved correct. Refusing to be discouraged by the objections, Fourier developed his treatment of the heat conduction problem into one of the classical triumphs of physics, and incidentally developed his use of trigonometrical series in the solution of his differential equations of conduction. All 'that is well brought out by Dr Herivel, who has deliberately concentrated on Fourier as a physicist and a man, on the grounds that Fourier the mathematician has been well covered. But I would have been grateful had some of Fourier's mathematics been included in the same volume because although, even if we agree with Dr Herivel that "It would be unrealistic to strike a balance between Fourier the man and Fourier the savant", the interplay of mathematics

and physics in Fourier's achievements must be a phenomenon of great interest. But, even regarding Fourier as a physicist alone, it is hard to understand why there is no reference in the book to Fourier's invention of the method of dimensions, which almost every subsequent physicist has used (Maxwell, for example) and which is a sure criterion of Fourier's insight.

Dr Herivel almost conveys a sense of disappointment that conspicuous though Fourier's civic role was it "can merit no more than a footnote in the history of the period whereas both as a physicist and as a mathematician he was undoubtedly one of the major figures of the nineteenth century and beyond". But what is really remarkable, as in the case of Handel, is that interspersed among the passages of a tempestuous and worldly career, Fourier should have achieved such heights of contemplativ. creation. Men of science who still have to battle with administrators who are reluctant to regard them as equals may point with gratitude to the example of Fourier. And their gratitude may be extended to Dr Herivell for this, within its limits, excellently annotated and documented account of Fourier and his times.

Tissue connection

Connective Tissue: Macromolecular Structure and Evolution. (Molecular Biology—Biochemistry and Biophysics, vol. 19.) By Martin B. Mathews. Pp. xii+318. (Springer: Berlin and New York; Chapman and Hall: London; May 1975.) £15.30.

THIS monograph, the 19th in the series *Molecular Biology—Biochemistry and Biophysics,* is an excellent review of the macromolecular structure and evolution of connective tissue. During recent years the advances in this field have been substantial indeed and this is the first time that an attempt has been made to consider this vast, widely scattered literature in the context of evolution and taxonomy.

The general construction and arrangement of the book are admirable. The first six chapters, amounting to about half the text, consist of a concise survey of the biochemistry of the macromolecular constituents of connective tissue: collagen, elastin, structural glycoproteins and polyanionic proteoglycans. The emphasis lies very much on the description of primary levels of organisaton, although relevant conformational data are mentioned and occasionally described. Each chapter also presents a most comprehensive account of the comparative biochemistry of these macromolecules, leading to considerations of molecular phylogeny. These considerations are,

however, hampered by the rather limited amount of structural information, particularly concerning amino acid sequences, which is now available. The coverage of collagen and glycosaminoglycans is extensive and this makes even more noticeable the somewhat limited space given to the discussion of elastin. This is unfortunate as the structure of that protein must still be regarded as controversial and the reader would have been helped by a more critical appraisal of the literature.

Professor Mathews' own particular interests—changes in cartilage biochemistry with embryonic development, maturation and ageing, and the taxonomic distribution of glycosaminoglycans—are presented in the next three chapters These contain separate sections for individual tissues, among which particular emphasis is given to cartilage, bone and notochord. Apart from their evolutionary significance, they are a mine of information and contain some original, unpublished work from the author's laboratory.

The last chapter is concerned mainly with the molecular properties and interactions of collagen and proteoglycans and because it encompasses so many facets of the subject, it should appeal to readers from many other disciplines.

The style is direct and unpretentious and the author admirably combines erudition and lucidity of exposition. This book will be most welcome to those working in this field.

A. Serafini-Fracassini

Environmental mechanics and thermodynamics

Fluid Mechanics and Thermodynamics of our Environment. By S. Eskinazi. Pp. xv+422. (Academic: New York and London, February 1975.) $26 00; £12.50.

IN comparison with other books on the motion of fluids by the same author, this one is a little disappointing in its production and layout. According to the preface the word 'environment' takes a variety of meanings and it is a word which is certainly made use of far too often in the text; usually, the more precise term 'atmosphere' or 'ocean' would suffice in its stead. The book is aimed at engineering students interested in air-borne and water-borne pollution but, though there is an obvious need for a concise introductory text which covers this field, this presentation leaves one with the impression that it is partly an attempt to jump on the current bandwagon of concern over man's use of the Earth as a waste disposal unit. The diagrams are the worst feature of the book and are sometimes amateurish in style. Their reproduction is usually on so small a scale that details tend to be obscured.

On the good side, the book is a useful introduction to both physical meteorology and oceanography, necessary parts of the curriculum of anyone studying pollution. Usually, these two subjects are treated separately but here the author has brought together their simpler aspects in one unified text and has attempted to present the material in a manner not requiring formal courses in fluid mechanics and thermodynamics as prerequisites On the whole he has succeeded although a good background in physics and mathematics is essential for a full understanding of the content of the book. Nevertheless, in one or two places, the reader is plunged into discussions of turbulent flows without the benefit of earlier treatment of simpler laminar models. Although it can be argued that flows in the atmosphere are rarely laminar, the difficulties of the treatment of the motions of a real atmosphere are often daunting to an undergraduate with no training in hydrodynamics

The book should prove most useful to the experienced scientist or engineer who requires a knowledge of the rudiments of meteorology and oceanography. It may also find some application in courses in geophysics which are not solely confined to a study of the solid Earth.

C. W. Titman

Non-earth science

Planetary Geology. By Nicholas M. Short. Pp. xv+361. (Prentice-Hall: Englewood Cliffs, New Jersey, April 1975.) $17.95.

THE content of this book is both narrower and wider than the title *Planetary Geology* would imply. The only extraterrestrial body to have been visited by man, extensively sampled and studied with a variety of on-site instruments is, of course, the Moon; and so it is hardly surprising that over 50% of the book should be devoted to lunar studies. Making allowances for the general introduction, the summing up and a chapter each on meteorites, the origin of planets and general aspects of the Solar System, only 15% of the book remains for "Mars, Venus, and the planets beyond"—a fair reflection of the present state of knowledge.

On the other hand, the term 'geology' is plainly inadequate, even for the seven chapters which summarise our physico-geological knowledge of the Moon. Dr Short is an employee of NASA and has used the perspective this gives him to write a book which sets the science firmly against the operational and methodological background of lunar exploration. This "inquiry- and problem-oriented" approach gives the book a distinctive flavour which I found a welcome change from the artificial world of the average text. The inclusion of wider contextual details may lead to some cavilling among academic purists, but the effect is surely to enhance rather than detract from the purely scientific story.

In a fast-moving field such as this, rapid dating of material is an obvious and serious problem. This book takes account of work caried out by to July 1974 (a remarkable achievement under present circumstances), which makes it the most up-to-date and comprehensive text on the subject now available. It would be a pity if it were allowed to age, for it forms an excellent foundation upon which to build later results; and I hope that Dr Short will be granted both the facilities and inspiration for frequent updating. Not that he is a stranger to revision. The first draft of this text was used and evaluated by students and then extensively rewritten in the light of the criticism and suggestions received. This is a procedure which many other authors could well adopt with benefit to themselves and advantage to their suffering readers.

Peter J. Smith

IAEA Publications

Thermodynamics of Nuclear Materials 1974

Volume 1

Proceedings of a Symposium, Vienna, 21-25 October 1974   £12 (£12.37)

Formation of Uranium Ore Deposits

Proceedings of a Symposium, Athens, 6-10 May 1974   £16.40 (£16.86)

Atoms, Molecules and Lasers

Lectures presented at the International Winter College, Trieste, 17 January-10 April 1973, organized by the International Centre for Theoretical Physics, Trieste.
£16 (£16.46)

Prices in brackets include postage

Post your orders to HMSO, PO Box 569, London SE1 9NH. Government publications and those of certain International Organisations can be obtained from HMSO Bookshops/Agents or booksellers.

HMSO BOOKS

Chromosomin re-emerges from limbo

Acidic Proteins of the Nucleus. (Cell Biology: A Series of Monographs.) Edited by Ivan L. Cameron and James R. Jeter. (Academic: New York and London, December 1974.) $28.50; £13.70.

CHROMOSOMIN was described by Edgar and Ellen Stedman about 35 years ago as one of the three major components of chromosomes, the other two being nucleic acids and histones. They postulated that it could have a structural role in the chromosome whereas the histones were gene regulators. Partly because of this speculation and partly because chromosomin proved very refractory to handle, it disappeared into the limbo of half-forgotten things for a quarter of a century (during which time the histones were studied intensively) but re-emerged about 10 years ago, renamed the non-histone or acidic chromosomal proteins, as a result of experiments which suggested that they, and not histones, might contain regulatory elements.

These proteins are complex. Upwards of 50 individual peptides (not all of which are strictly acidic) can be demonstrated and many, perhaps most, are probably not involved in regulation. Like a gold strike, this area is, therefore, full of promise, challenge and excitement; but for every worthwhile discovery, there are many disappointments. Monographs on the subject, for this reason, have been slow to appear and this is one of the first to be devoted entirely to the acidic proteins of the nucleus. It is, therefore, of special timeliness and interest.

It has 10 contributions. Vincent Allfrey first discusses control in prokaryotic and eukaryotic systems, drawing analogies between them; he also outlines preliminary experiments on the isolation of specific DNA-binding proteins. Gordhan Patel then presents a detailed review of methods for the isolation and fractionation of acidic proteins, which is followed by an account by LeSturgeon and Wray of their work with phenol-soluble acidic proteins. The fourth chapter, by Lewis Kleinsmith, discusses modification by phosphorylation and the possible role of this in modulating nuclear function. Chapters 5 by Bruce McGunn and 6 by LeSturgeon, Totten and Forer are concerned mainly with the nucleoproteins of Physarum polycephalum. Chapter 7, by Berendes and Helmsing deals with the non-histone proteins of Dipteran polytene nuclei and is of particular interest since it brings together some biochemical findings and the morphological evidence about gene activity. The chapter, by the editors themselves reviews changes which have been reported in acidic proteins during cell growth. Tom Spelsberg devotes a chapter to a consideration of the role of nuclear acidic proteins in binding steroid hormones and, finally, Stewart Gilmour reviews the evidence that acidic proteins are involved in gene regulation in eukaryotes and discusses some of the models which have been proposed.

It is inevitable that any contemporary account of this field should be diffuse but gene regulation in eukaryotes is one of the greatest remaining challenges to the biologist and the acidic proteins of the cell nucleus are clearly implicated in it. The editors (Ivan Cameron and James Jeter) have managed to obtain contributions from several of the most experienced people in the field and the volume provides a reasonably comprehensive, up-to-date summary. It can, therefore, be recommended to laboratories in which work of this kind is being undertaken or contemplated.   **John Paul**

announcements

Awards

The 1975 **Hewlett-Packard-Europhysics Prize** for solid state physics has been awarded to **V. S. Bagaev, L. V. Keldysh, J. E. Pokrovsky** and **M. Voos.**

H. S. Schwartz has been awarded the **Johananoff International Fellowship** for advanced biomedical studies.

The Pontifical Academy of Sciences has awarded the **Pius XI Gold Medal** to **S. W. Hawkins** for his work on membranes.

Miscellaneous

Lunar Research. NASA is continuing to encourage and support research on all aspects of lunar science. Scientists with new ideas, techniques and special capabilities are encouraged to participate. NASA's lunar programmes include: experimental and theoretical research on lunar materials, lunar data analysis and synthesis, and supporting research and technology.

Further information from Dr E. A. Flinn, Director, Lunar Programs Office, Code SM, NASA, Washington, DC 20546.

International meetings

September 2–6, **Function of living plant collections in conservation,** Kew (Christine Brighton, Royal Botanic Gardens, Kew, Richmond, Surrey, UK).

September 9–11, **Applications and economics of remote sensing systems,** Silsoe, Bedfordshire (Mr T. S. Bell, ADAS, MAFF, Block B, Government Building, Brooklands Avenue, Cambridge, CB2 2DR, UK).

October 22, **Applications of analytical techniques to industrial effluents,** Colchester (Analytical Division, Chemical Society, 9 Savile Row, London W1X 1AF, UK).

October 26–November 1, **Alcoholism and drug dependence,** Sao Paulo, Brazil (International Council on Alcohol and Addictions, Box 140, 1001, Lausanne, Switzerland).

Reports and publications

Great Britain

Potato Marketing Board. Sutton Bridge Experimental Station Annual Review 1974 71. Summary of Investigations Undertaken in 1973/1974. Pp. 7. (London. Potato Marketing Board, 50 Hans Crescent, SW1, 1975) *gratis* [106
Half the Loaf A Study on the World Food Crisis. By Colin Pritchard Pp. 41 (Edinburgh The Saint Andrew Press, 1975.) 30p net. [116
Philosophical Transactions of the Royal Society of London. B· Biological Sciences. Vol. 270, No 908: Excitable Membranes. A Discussion organized by R D. Keynes, FRS. Pp. 295–559. (London. The Royal Society, 1975) UK £10 70; Overseas £11.20 [116
The Forestry Commission's Objectives. Pp. 16 (Edinburgh: Forestry Commission, 231 Corstorphine Road, 1975.) [126
University of Bristol. Department of Agriculture and Horticulture. Report of the Long Ashton Research Station (The National Fruit and Cider Institute), for 1974. Pp. xxii + 220 + 7 plates. £2. An Introduction to Modern Cider Apple Production. By R R. Williams. Pp. 34. 50p (Long Ashton, Bristol· Long Ashton Research Station, The University, 1975.) [136
Report of the Tropical Products Institute, 1972/1974 Director P. C Spensley. Edited by Melba Kershaw and J B Davis. Pp. v + 96 + 12 photographs. (London· Tropical Products Institute, 127 Clerkenwell Road, EC1, 1975.) 90p net. [136
Ministry of Overseas Development. Annual Report of the Directorate of Overseas Surveys for the year ended 31st March 1973 Pp. 59 + 5 maps (London· HMSO, 1975.) £2 net. [166
The Baby Killer. (A War on Want investigation into the promotion and sale of powdered baby milk in the Third World). By Mike Muller. Second edition, with Appendix Pp. 23. (London: War on Want, 467 Caledonian Road, N7, 1975) 40p. [196
British Overseas Trade Board Export Handbook: Services for British Exporters Eighth edition. Pp. 192. (London· British Overseas Trade Board, 1 Victoria Street, 1975.) *gratis* [206
The Agricultural Research Service. Pp. 36. (London Agricultural Research Council, 169 Great Portland Street, W1. 1975.). [206
Sun at Work in Britain, No 2, May 1975. Pp 46 (London· International Solar Energy Society, c/o The Royal Institution, 21 Albemarle Street, W1, 1975) £1; $3. [206
Microbiological Research Establishment. Abstracts of Work Published in 1974 Pp. 15. (Porton, Salisbury, Wilts· Microbiological Research Establishment, 1975.) [236
Philosophical Transactions of the Royal Society of London. A· Mathematical and Physical Sciences Vol. 278, No. 1284 Baroclinic Waves in a Container with Sloping End Walls. By P. J Mason. Pp 397–445. UK £2 10, Overseas £2 20 Vol. 278, No. 1285: Marine Geology of the Rockall Plateau and Trough. By D. G Roberts. Pp. 447–509. UK £4 95; Overseas £5 10 (London The Royal Society, 1975.) [236
The Tidal Threat· East Head Spit, Chichester Harbour. By S. A. Searle Pp 24. (West Wittering, Chichester The Dunes Group Distributed by The National Trust, Polesden Lacey, Dorking, Surrey) £1. [236
Royal Greenwich Observatory—Report 1974 Pp 43 + 4 plates 40p net. Royal Greenwich Observatory Illustrated. Pp. 25 30p. (Herstmonceux Castle, Hailsham, Sussex Royal Greenwich Observatory, 1975.) [236
Proprietary Association of Great Britain. Annual Report 1974/1975 Pp. 12 (London· Proprietary Association of Great Britain, Victoria House, Southampton Row, WC2, 1975.) [236
Cancer Research Campaign. 52nd Annual Report 1974, Incorporating Research Supported During 1975. Pp. 144 (London· Cancer Research Campaign, 2 Carlton House Terrace, SW1, 1975.) [246
Bulletin of the British Museum (Natural History). Entomology, Vol 32, No. 3· Revisional Notes of African Charaxes, Palla and Euxanthe (Lepidoptera Nymphalidae), Part X. By V. G L. van Someren Pp. 65 – 136 + 19 plates. (London British Museum (Natural History), 1975.) £13. [246
John Innes Institute. Sixty-Fifth Annual Report, 1974 Pp. 151. (Norwich, Norfolk· John Innes Institute, Colney Lane, 1975.) £1.50. [266
Code of Practice for the Large Scale Storage of Fully Refrigerated Anhydrous Ammonia in the United Kingdom Pp. 38. (London: Chemical Industries Association, Ltd , 93 Albert Embankment, SE1, 1975.) £2.50. [266
Department of Industry Technology and the Environment Reports from Scientific Councellors Overseas, No 8· Monitoring of Pollutants Pp 34. (London: Department of Industry, Abell House, John Islip Street, SW1, 1975.) [276

BP Statistical Review of the World Oil Industry 1974 Reserves/Production/Consumption/Trade Refining/ Tankers/Energy. Pp. 24. (London: The British Petroleum Co., Ltd., 1975.) [306
Asbestos· Its Special Attributes and Uses. Pp. 32. (London Asbestos Information Committee, 2 Old Burlington Street, W1, 1975) £1.30 [306
Smith Kline and French Foundation Twelfth Annual Report, 1974. Pp. 9. (Welwyn Garden City, Herts Smith Kline and French Foundation, 1975) [306
Bulletin of the British Museum (Natural History), Geology, Vol. 26, No. 1· The British Lower Jurassic Species of the Bivavle Genus Cardinia. By C P. Palmer. Pp. 1 – 44 + 5 plates. (London. British Museum (Natural History), 1975.) £3.30 [306
Devon Trust for Nature Conservation. Salcombe Hill, Sidmouth—Nature Trail Guide. Pp. 16 (Exeter· Devon Trust for Nature Conservation, 1975) 20p.[306
Science Research Council. Non- Newtonian Flow· a Review of Problems and Research. Pp. 53. (London. Science Research Council, State House, High Holborn, 1975.) *gratis* [306
Philosophical Transactions of the Royal Society of London B. Biological Sciences Vol. 271, No. 910 The Enigmatic Animal Opabinia regalis, Middle Cambrian, Burgess Shale, British Columbia. By H. B. Whittington Pp. 1 – 43 + plates 1 – 16. UK £4.05, Overseas £4 20. Vol. 270, No. 909 Studies of the Post-Glacial History of British Vegetation. XVI. Flandrian Deposits of the Fenland Margin at Holme Fen and Whittlesey Mere, Hunts. By Sir Harry Godwin and Vishnu-Mittre Pp 561–608. UK £2.10; Overseas £2 20. (London. The Royal Society, 1975.) [306

Other Countries

Australia· Department of Defence. Australian Defence Scientific Service. Materials Research Laboratories Report 620 The Determination of Colorimetric Values of Colours to BS 381C. By R. J. Hancox and L. A. Hill. Pp. 12 (Ascot Vale, Victoria: Materials Research Laboratories, 1974.) [186
Det Danske Meteorologiske Institut. Meddelelser Nr. 25 An Operational Spectral Semi-Implicit One Layer Model By B P. Byrnak. Pp. 48. (Kobenhavn. Danske Meteorologiske Institut, 1975.) [186
Argentino. Direccion Nacional del Antartico. Contribucions del Instituto Antartico Argentino. No. 154. Resultados Planctologicos de la Campana Oceantar I—I Quetognatos Por Estela Olga Dinofrio. Pp 62. No. 164: Estratificacion del Complejo Bentonico de Invertebrados en Puerto Paraiso (Antartida). Por Zulma J. A. de Castellanos Pp. 30. No. 165 La Superficie del Esmalte de la Foca de Weddell (Leptonychotes Weddelli) Tratada con Acido Lactico. Por Abraham Abramovich. Pp 11 No. 167 Iluminacion Natural en Bahia Esperanza—I Antartida Argentina. Por M. F. de Mattiello, F A. Mattiello and E. A. Buccella. Pp 15 No. 168· La Glandula Pineal del Pinguino Papua (Pygoscelis Papua) Contenido de Serotonina y Melatonina Por R. S. Piezzi, M. R. Gallardo, M. H. Burgos, J Carlos de Rosas ans L. Gutierrez Pp. 11. No. 170 Estimacion de Volumenes y Espacios Iluminacion Natural en Bahia Esperanza (II) Por M. L. F de Mattiello y F A Mattiello. Pp. 19. No. 176 Contribucion al Conocimiento de Los Parasitos de la Fauna Antartica. Parte I Clavellopsis Tomoim n. sp. un Nuevo Copepodo Parasito de Trematomus Bernacchii Boulenger Por T Stadler. Pp 11. (Buenos Aires· Instituto Antartico Argentino, 1973 and 1974) [196
Parliament of New South Wales Report of the Department of Agriculture for the year ended 30 June 1973. Pp. 208 (Sydney. Department of Agriculture, 1974.) [196
Académie Royale de Belgique. Annuaire pour 1975. Pp 355. (Bruxelles. Académie Royale de Belgique, 1975.) [236
World Health Organization, Geneva. Manual on Practical Entomology in Malaria Part 1 Vector Bionomics and Organization of Anti-Malaria Activities. Pp. 160 Part 2· Methods and Techniques. Pp. 191. Prepared by the WHO Division of Malaria and Other Patasitic Diseases. (Geneva· WHO, London HMSO 1975) Part 1 Sw fr 21, Part 2 Sw.fr.24. [236
Population Reference Bureau, Inc Population Bulletin, Vol. 30 No. 1 Africa and Its Population Growth. Pp 27. Population Bulletin, Vol 30. No. 2. Literacy and World Population Pp 29. (Washington, DC. Population Reference Bureau, Inc., 1975.) [246
Australian Academy of Science, Report No 18. Report of Working Group on Diet and Coronary Heart Disease Pp 72. (Canberra Australian Academy of Science, 1975.) [246
US Department of Commerce National Oceanic and Atmospheric Administration. Key to Geophysical Records Documentation No. 4 Marine Geophysical Data Catalog—1975 Pp ii + 371 (Boulder, Colorado National Geophysical and Solar-Terrestrial Data Center, 1975 For sale by US Government Printing Office, Washington, DC.) $5 25. [246

August 28, 1975

See how they run

THE chart in the centre of this week's issue of *Nature* is not, you will be disappointed to hear, the result of furtive investigations by the staff under trying conditions. None of our sub-editors was dispatched with binoculars to phone-boxes in Whitehall to catch glimpses of faceless men doing a quick sprint out of their ministry to black government cars waiting to take them to assignments in other ministries. No laser beams were trained on the curtained windows behind which whispered conferences were being held. All the information is in the public domain. The research was no more exciting than several days of delving through annual reports, some telephone calls and a few fivepenny bus rides to pick up official documents. And in a way the product of the research isn't exciting either. Old hands on the London scene will look at the chart and say that there is nothing on it they didn't know about already—and that there is enough inter- and intra-departmental committee structure in Whitehall to convert our simple little picture into a veritable cat's cradle of inter-connections.

Indeed the old hands are right. The proliferation of working parties, study groups, panels and sub-committees is unending and we could have easily devoted six months of our time to trying to put together a coherent picture of this fine detail. But how many are there who can even be called old hands? The majority of British scientists will probably find that even our elementary chart reveals quite a lot they hadn't realised before. Who runs Kew? Harwell? What ministry pays for space research? How much is spent on defence? How are we represented in Europe? At UNESCO?

It is, of course, almost in the very nature of a central bureaucracy almost exclusively concentrated in one city,

that those on the outside will take a Kafka-esque view of it. Contact with the outside world tends to be through the trusted advisers or council members; criticism from the press is greeted with stony silence, communications from outside tend to leave the individual feeling impotent. And yet the castle is fairly easily penetrable to those who take along a map, and most of its influential inhabitants have been scientists themselves. Moreover, because of the perennial problem that central bureaucracy has in accurately judging the feelings of those at the grass roots, anyone with the ability to communicate with the right bureaucrat at the right time can have a disproportionate influence—for good or bad.

Hardly anyone on this chart could be said to have got there by anything resembling a democratic process, and by the same token hardly anyone could be removed by anything resembling a democratic process. We are not about to advocate annual elections to councils and committees; what we do urge, however, is a much wider understanding of the rudiments of science policy-making, and maybe this chart will help.

Only one committee which we wished to list has had to remain ex-directory. The Defence Scientific Advisory Committee, we have been told (not by its chairman), prefers to remain anonymous, partly for fear of student action against its members. In view of the immense expenditure on defence research and the almost complete ignorance in the academic and student world of defence matters, anonymity can only serve to create further alienation.

● Extra copies of the chart are available. See page iii for details.

Earthquakes cause cancer: official probe shock

THE annual British Association meeting is upon us again, and so is the silly season for newspapers. Here are a few simplistic headlines science could well do without in the coming week.

Coffee-drinking causes cancer, scientist says

British astronomy leads the world

Concorde's effects harmless—government scientist

Cancer drug hopes

Sex at 80 keeps you fit—scientist

Earthquakes threaten North Sea oil

Tea-drinking causes cancer

Concorde 'will end world as we know it'—environmentalist

British astronomy in doldrums

New X-ray star no hazard to health

Scientists create life in laboratory

Can readers of *Nature* think of any others?

Weather watchers

As fewer and fewer agricultural regions have supplied increasing amounts of the food consumed by the countries where population growth is greatest, there has been growing concern about how the climate of the next few decades may affect world agricultural productivity. Henry Lansford, of the National Center for Atmospheric Research, Boulder, Colorado, reports.

ROBERTS

SOME atmospheric scientists, projecting trends of the past 30 years or so, foresee steadily decreasing temperatures accompanied by greater climatic variability—more droughts, floods, unseasonable cold spells, and other extreme events that certainly would reduce agricultural productivity, at least in the regions where they occurred. Professor Hubert Lamb, of the University of East Anglia, and Professor Hermann Flohn, of the University of Bonn have warned that this sort of climatic change seems to be in progress, together with Dr Reid Bryson, of the University of Wisconsin, and Dr Valter Orr Roberts, who formerly headed the National Center for Atmospheric Research (NCAR) and is now with the Aspen Institute for Humanistic Studies.

Other climatologists maintain that the climatic trend of recent years does not necessarily represent a long term change, but should be viewed as a fluctuation that may halt or reverse itself at any time. These climatological conservatives maintain that our understanding of the mechanisms of climatic change is so rudimentary that no sound basis exists for assuming that a downward trend in temperature will continue in the future. Among these conservatives are Dr B. J. Mason, who heads the British Meteorological Office, and people like Dr J. Murray Mitchell of the National Oceanic and Atmospheric Administration (NOAA), and Professor Helmut Landsberg, of the University of Maryland.

But even these conservatives are worried about the impact of climatic variability on world food supplies. The potential consequences of droughts and other climatic anomalies are greater than ever before because of present low levels of grain reserves, increasing use of semi-arid and other marginal lands for farming, and expectations of continuing high yields

from the new "Green Revolution" crops that are highly responsive to generous supplies of water and fertiliser.

During the past year and a half, atmospheric and agricultural scientists, as well as specialists in fields such as economics, international development, law and political science, have gathered for a succession of international conferences designed to identify some of the major problems of climate–food–social interactions and to try to develop new research strategies for attacking those problems effectively. The meetings have included:
● A conference on "Weather and Climate Change, Food Production and Interstate Conflict" held in New York City by the Rockefeller Foundation in January 1974
● A workshop on "The Impact on Man of Climate Change" held at the University of Bonn by the International Federation of Institutes for Advanced Study (IFIAS) in May 1974
● A conference on "World Food Supply in Changing Climate" held at Sterling Forest, New York, in December 1974 under the joint sponsorship of the American Society of Agronomy and the Aspen Institute for Humanistic Studies
● A pair of workshops, one on the policy implications of food and climate interactions and the other on the design of a study of the social, political, economic and ethical impacts of drought, held in West Berlin by IFIAS and the Aspen Institute in February 1975
● A conference on "Climate Change, Food Production and Interstate Conflict" held by the Rockefeller Foundation at Bellagio, Italy, in June 1975.

Although the participants at these meetings disagreed about a great many things, there seems to be fairly general agreement that the vagaries of climate do indeed represent a serious threat to world food supplies in

these precarious times of rapidly growing demand for food, low world grain reserves and increasing dependence of many heavily populated nations on a few regions of high agricultural productivity, usually halfway around the world from the regions of high consumption, for their food supplies.

The conclusions, recommendations, and resolutions that have come out of these conferences have ranged from the strident to the cautious. The IFIAS statement that emerged from the Bonn meeting, for example, said that:

"The nature of climatic change is such that even the most optimistic experts assign a substantial probability of major crop failures within a decade. If national and international policies do not take such failures into account, they may result in mass deaths by starvation and perhaps in anarchy and violence that could exact a still more terrible toll."

With more reserve and less rhetoric, the participants in the recent Bellagio Conference came to much the same conclusion. They agreed that:

". . . there is some cause to believe —although it is far from certain— that climatic variability in the remaining years of this century may be even greater than during the 1940–70 period", and concluded that "greater climatic variability than that of the last two decades could cause major crop failures quite beyond the current capability of agricultural science and technology to control or mitigate."

Although these climate–food meetings have not resulted in any solid consensus among atmospheric and agricultural scientists about just what the climate is going to do and how it will affect food supplies, much less about what international policies and mechanisms will be needed to cope effectively with conflicts that may develop among nations as a result, a few common conclusions seem to be
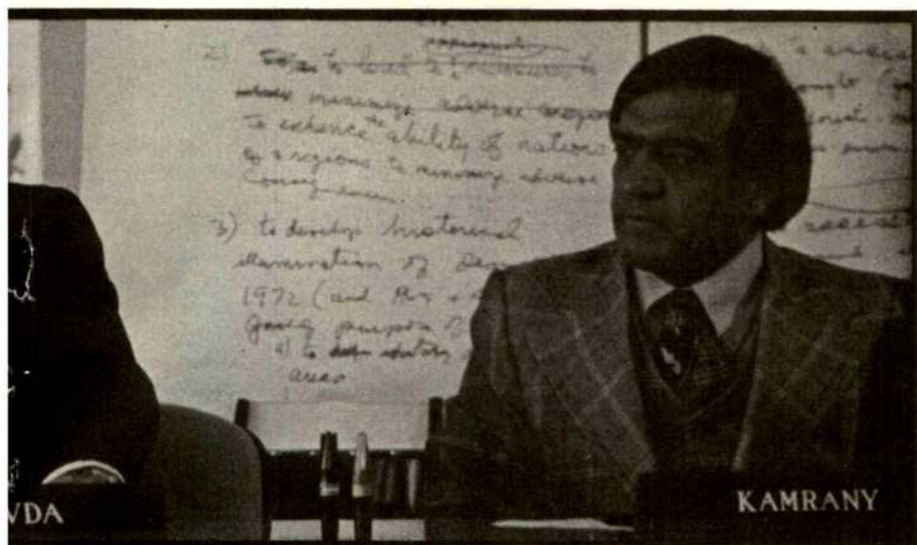
KAMRANY

Walter Roberts, Nake Kamrany and Viktor Korda in Berlin.

emerging from all the discussion and debate.

As far as the climate itself is concerned, there is fairly general agreement on two points:

● The climate of the first half of the twentieth century was considerably warmer than the average of the past 1,000 years, which suggests the possibility that cooler weather can be expected to follow.

● The average global temperature, which gradually increased during the first half of the century, has been decreasing during the past quarter of a century, at least at latitudes above 55 °N. This cooling was between 2 and 3 °C for some northern locations such as Iceland. Lamb points out, however, that the length of the growing season in England increased by two to three weeks during the first half of the century and has dropped back by about two weeks since 1945.

There is considerable disagreement about whether or not the cooling trend is likely to continue. One school of opinion, which includes Bryson, Flohn, Lamb and Roberts, sees a strong probability that the downturn in temperature that has occurred since 1950 will continue for at least another two or three decades. Other climatologists such as Mitchell and Landsberg maintain that the causes and mechanisms of climatic change are not understood well enough for anyone to make such a prediction with any assurance of success.

Although a number of the scientists who anticipate a global downturn in temperature participated in the Bellagio Conference, the group as a whole took a cautious view of the question of global cooling. The conference participants concluded that:

"It is not certain that the decline in Northern Hemisphere temperature will continue; it may halt or reverse itself. Even if the global average temperature were to decrease steadily over the next 20 years, it is unlikely that it would drop as much as one degree Celsius."

But they went on to point out that: "temperature change *per se* is not the most serious climatic threat to food production. The possibility of increased climatic variability, particularly in distribution of precipitation, is of greater concern."

The question of variability is another subject of controversy. Bryson and others believe that the cooling trend in the high latitudes of the Northern Hemisphere has changed large scale atmospheric circulation patterns by increasing the temperature contrast between tropical and polar regions. They believe that this has produced increased climatic variability—a higher incidence of droughts, floods, unseasonable cold weather and other extreme events that have characterised recent bad crop years such as 1972 and 1974.

Whether or not the climate is growing more variable, even the climatological conservatives such as Landsberg agree that climatic variability represents a continuing threat to uniform crop yields in many agricultural regions. Although the Bellagio Conference paticipants did not conclude that the climate is becoming increasingly variable, they agreed that "climatic variability—region by region and from year to year in particular regions—is and will continue to be large."

In discussing problems of climate and food, atmospheric scientists have frequently referred to the climate of the first half of this century as "benign," implying, perhaps unintentionally, that it provided the best possible growing conditions for crops everywhere. But at Bellagio the agricultural scientists pointed out that this is not so. The frequency of droughts in the Soviet Union increased sharply during that period. In parts of the midwestern United States, summer temperatures have often been too hot for

optimum yields from some crops that are grown there. A general cooling trend, while shortening the growing season in some northern regions, might result in a compensating increase in crop yields in regions further south. Professor Lamb has suggested that, in discussions of climate change, it would be wise to avoid using the words "benign" and "normal," as they both imply value judgments that are subject to considerable argument.

After listing some of the impacts that a cooling trend might have on agriculture—changes in distribution of precipitation, shorter growing seasons in high latitudes, and cooler growing temperatures in some regions—the Bellagio participants admitted that:

"Some effects of such climatic changes would be beneficial to agriculture and others would be detrimental, but the net effect, for the whole world or for any particular region, is not known."

To remedy this lack of knowledge of the agricultural impact of future climatic developments, it was recommended that a set of 'scenarios' specifying plausible future climatic sequences be developed and that the impact of each possible climatic future on agricultural production, the world food situation and international relations be assessed as precisely and quantitatively as possible.

Regardless of the changing casts of characters, different approaches, and specific conclusions that have characterised the recent climate–food meetings, one grim theme emerges from all of them. Whether or not any long term change to cooler temperatures or increasing climatic variability is in progress, there is a high probability that major regional and world food emergencies will occur during the next decade or two as a result of serious reductions in crop yields or major crop failures caused by climatic anomalies.

A number of possible research and policy strategies have emerged that could contribute to effective national and international responses to such emergencies.

It seems that the atmospheric scientists should accelerate their efforts to determine whether or not it is possible to predict climatic conditions a season or more in advance, as well as examining ways in which existing climatic information can be made more useful in coping with agricultural problems. An improved understanding of the workings and interactions of the five physical elements of the total climatic system—atmosphere, hydrosphere, cryosphere, lithosphere and biosphere—is badly needed. Both theoretical and empirical approaches to problems of climate change should be pursued. Agricultural scientists should

assess the potential impact of various climatic anomalies on various crops, particularly the new high yield varieties, and should consider plant breeding strategies, as well as techniques of crop, soil and water management, that can reduce the impact of climatic stresses on agricultural productivity. Special attention should be given to the role of soils in mitigating the impacts of drought, and to the improvement of dryland-farming practices, as more and more semi-arid lands are brought under cultivation.

Application of new and existing agricultural technology to improvement of crop yields in regions that supply food for their own consumption seems to be at least as valuable in the long run as striving to continue to increase yields in exporting regions such as the United States and Canada.

Policy makers must assess national and international policies on agriculture and food in the light of the probability that major food shortages, affecting large regions if not the entire world, can be expected to result from climatic stresses over the next few decades. Although years of bad climate such as 1972 may not necessarily reduce total world food production, they clearly have the potential for disrupting world food trade patterns and raising prices to the extent that people in some regions will go hungry not because there is no food, but because it costs so much that they cannot afford to eat properly. The potential is great for conflict among nations as well as for human suffering in the regions that are affected directly by climatic anomalies.

There does not seem to be any miraculous 'technological fix' waiting in the wings to save the day. Agricultural technology probably can be applied to achieve additional increases of 100% or more in crop yields in some regions, primarily those where food consumption is highest, but such increases are not likely in most of the present major food-producing regions.

Climate forecasting will be useful only if workable alternatives are available so that the farmer can respond to the foreknowledge that a drought or a short growing season is imminent. Weather-modification technology may have the potential for improving crop yields in some regions under some conditions, but it has not been demonstrated that cloud seeding can do much, if anything, to remedy major climatic anomalies such as large scale droughts.

Science and technology certainly have appreciable contributions to make to solutions to our growing climate–food–society problems. But the evidence that has emerged from the recent conferences indicates clearly that the responsibility for establishing a framework within which scientific and technological knowledge can be applied effectively to solving the problems rests as much with the policy makers as it does with the plant breeders, the climatologists and the cloud seeders. □

Failing to take the point

P. A. MOHR

A small experiment now taking place in Britain seems to be having results that warrant a little more attention from our drought-stricken sociological experts. I refer to the resistance of the Anglo-Saxon mind to metrication, a resistance which, whether inwardly sullen or sudden, is outwardly passive so long as our feet are not pushed to the wall. For the inevitably vociferous, the issue is seen in the emotive terms of an unimpeachable heritage threatened from without, and the City Editor of the *Sunday Telegraph* euphorically praises "the glorious indifference of the British people [to metrication]", himself excepted, presumably.

Because, here in the USA, metric measures are now unofficially and surreptitiously appearing in all sorts of odd corners, from the food and building industries to sports events, not to mention our completely ravished science, we are not indifferent to watching the unenthusiastic attitude of the British to losing their 2,000-year-old Roman measures. Admittedly, the decimal Roman measures became a little unmetrified after the UDI of Hengist and Horsa in a petrified British culture, but Anglo-Saxon we have staunchly remained for the 1,500 years since.

Are we now to throw over that measured tradition? Galileo learned, to his cost, the strength of tradition when, in the naive cause of truth, he tried fervently to upend it overnight. Pope Gregory's reform of the calendar was regarded by the English as even more of an occasion for cheating than the recent decimalisation of £.s.d. Tradition! Should we resist and drop metrication, except for odd corners of science that can easily be swept clean again; should we denigrate, admire or diplomatically ignore the resolution of South Africa in enforcing it by law in one fell swoop; or do we characteristically muddle on with a facade of creeping metrication that is merely a measure of creeping paralysis of will?

Certainly, the British Metrication Board, in urging the people of Britain to think metric for reasons of logic and utility alone, has completely misjudged the Anglo-Saxon mind. Those who oppose metrication see the ultimate argument quite clearly: metrication is alienation. Logic and possible future utility make no impact on the gloriously indifferent.

Can the Imperialists . . . (no, that won't do) . . . Can the opponents of metrication discover that the non–Anglo-Saxon world, by discarding multifarious old measuring systems, has impaired its heritage? It might make for an interesting discussion; but surely many would prefer to regard tradition as something not merely inherited, but to be fashioned for those who will inherit it, hopefully improved, from the living. Otherwise we would still be sticking to the measuring system of Boadicea, without the benefit of those delightfully duodecimal Roman "uncias" of length and weight. (British readers, now perhaps only able to afford their tobacco by the gram, may be interested to know that in the USA "one pound" of pipe tobacco has shrunk to 12 ounces, in precise conformity with the original definition, but not, alas, out of any respect for tradition.)

Need we go metric? Cannot measuring systems of the world co-exist as do languages? Is there a good case for Britain and the USA to drop, or stop, the whole costly and unwanted experiment and re-trench? As far as I am aware, the token metrication of Britain has barely entered everyday thought, estimation and judgment. One does not see Mr. Wilson, one of Britain's better known pragmatists, contemplating his bowl in centimetres and °C.

If metrication, an abomination of a word redolent of graduation in dietetics, really is desirable for us Anglo-Saxons, then it will not stem from the will of the people in their present mood. Either those in authority must coerce, or those favouring the change must be seen to step aside from the appointed bureaucrats of Whitehall and Washington, and start cutting ice, or, for those who disdain the methods of Sam Adams and the founders of the American Revolution, advertise the fact in the manner of the huge, state sign on the main Boston–Montreal highway: "Barre, 100 kilometers".

Maybe we can continue along the broad way, rather than take the narrow road of decision. But I fear that, in nautical parlance, we're tying ourselves in unfathomable knots.

international news

ALTHOUGH a crash research and development programme on solar energy has long been advocated by a small band of scientists and environmentalists, who see the Sun as a bountiful source of clean energy—and also by some Congressmen who see it as a useful source of votes—until recently the federal government has not seemed to share the enthusiasm. But there are now signs that the Energy Research and Development Administration (ERDA) has recast the priority of the solar energy programme it inherited from other government agencies earlier this year. Officials of ERDA are now publicly predicting that solar power could provide a very large contribution to energy needs in the United States in the twenty-first century.

The latest piece of evidence to that effect is contained in a report, published by ERDA earlier this month, in which the agency outlines an ambitious, 10-year research and development effort designed to reduce the costs of solar technologies and, it is hoped, to pave the way for their commercial introduction. ERDA states in the report that if the effort is successful, energy derived from the Sun could supply as much as 25% of the country's energy requirements by the year 2020.

Although it should be noted that the report is conspicuously devoid of cost figures—a factor which makes it difficult to compare ERDA's new proposals with previous government plans—solar enthusiasts are generally pleased that ERDA is slowly moving closer to their own predictions of what is possible (although it still has a long way to go). The new report, moreover, follows hard on the heels of the publication by

ERDA looks to the Sun

by Colin Norman, Washington

ERDA of an overall plan for energy research and development in which solar power is raised to the same level of priority as the breeder reactor and the thermonuclear power programme.

In spite of the generally optimistic tone of the report, however, ERDA is quick to caution that the potential contribution from the Sun will only be realised "if the costs of collecting and utilising solar energy can be reduced substantially"—in some cases by a factor of 100 or more.

The general approach outlined in the report is to press ahead as swiftly as practicable with demonstration projects, and to get early industrial participation in the effort to help pave the way to commercial introduction of the technology. A focal point for the programme will be a new solar energy research institute, a large laboratory which ERDA is planning to establish early next year which is expected to have a budget of about $50 million by 1980

The research programme is divided into three chief parts—direct thermal applications, such as solar heating and cooling; conversion of solar energy to electricity; and the development of fuels from plants.

Efforts in the first category are probably the most advanced. According to

a directive from Congress, ERDA plans to install up to 4,000 solar heating units in private homes and public buildings as part of a demonstration programme. (There are, however, unconfirmed rumours in ERDA and in Congress that the Office of Management and Budget—a powerful White House office similar in some respects to the British Treasury—will allow ERDA to install only 350 units.) The emphasis will shift to development of combined heating and cooling units in the late 1970s.

As for the conversion of solar energy to electricity, technologies to use wind power are closest to the demonstration phase. The National Aeronautics and Space Administration Agency is now building a small generating plant, and ERDA hopes to begin work next year on a larger unit. The commercial use of solar energy to generate electricity through photovoltaic cells will require some research breakthroughs to reduce the costs by a factor of at least 100, ERDA reckons, but demonstration plants with a capacity of between 1 and 5 MW are being planned for the mid-1980s. Similar sized plants for generating electricity from solar-produced steam are also planned for the mid-1980s, and in that case ERDA reckons that a cost reduction of between 50 and 70% will be required. The least advanced solar-electric concept at present is the idea of using ocean thermal gradients.

Finally, ERDA hopes to initiate a number of studies with industry in the late 1970s designed to explore the large scale use of plants and animal wastes to produce fuels such as alcohol and methane. □

NEW legislation on the conservation and use of mineral deposits was recently passed by the Supreme Soviet of the USSR and is to come into force on January 1, 1976.

The measures proposed do not, at first glance, seem particularly spectacular, and are concerned mainly with rational planning to reduce wastage, the introduction of new and more effective methods of ore dressing and petroleum processing, and improved methods of quality control, including the use of radioisotopes in both ferrous and non-ferrous metallurgy. The need for further prospecting for new beds and deposits and the improvement of prospecting methods is urged; this,

Legislation on use of Soviet minerals

from Vera Rich

incidentally, is regularly cited as a justification for the expenditure on the Soviet space programme, since, officially, Kosmos reconnaissance satellites are engaged, *inter alia*, in surveying mineral resources.

It is not, however, the measures themselves which attract attention so much as the urgency with which they are expressed. The Supreme Soviet "resolved: to consider as one of the

most important state problems the ensuring of rational, complex and economic use of mineral wealth and the intensification of its conservation within the aims of the further development of the socialist economy". In his address to the convocation, N. A. Tikhovov, Deputy Chairman of the Council of Ministers of the USSR, stated that in the past four years the Soviet national income has risen by 26%, the output of industry by more than 1.3 times, and real earnings by 19%. In order to continue thus to construct "the material and technical bases of a communist society", the rational use of mineral resources must be treated as a matter of great importance. Soviet

economic data are, traditionally, always presented in terms of percentages, and it is frequently difficult to refer them back to a base date, so as to calculate their meaning in real terms. Such figures for production as are readily available, however, give for the first half of 1975 240 million tons of oil, 141,000 million cubic metres of gas, 348 million tons of coal, 69:8 million tons of steel and 44 million tons o⁴ mineral fertilisers. Against this background of rapid expansion, the new laws seem to show a growing awareness that the mineral resources of the Soviet Union although vast, are still finite.

A *Pravda* editorial, dealing with the laws, stated: "Science is faced with great problems. Scientists are charged with creating new methods, techniques and technology which will allow mineral resources to be extracted without loss, with introducing them more fully into the national economy, and with ensuring the safety of mining operations". The Academy of Sciences of the USSR, the Academies of the Union Republics, the State Committee on Science and Technology and the various scientific research organisations as well as the Ministries concerned, will all be involved in implementing the new laws.

Meanwhile, surveying for new deposits continues. ☐

THE Baltic countries' scientific cooperation continues. Under the auspices of the Helsinki Convention for the prevention of pollution in the Baltic Sea, a meeting was recently held in the Swedish archipelago at which Swedish and Russian biologists studied each other's methods of intercalibration of measurements in marine biological experiments. The meeting was the second in a series (the first, last year, concentrated on chemical methods of measurement) which will continue next year with the additional participation of the other signatories of the Convention: Finland, Poland, the German Democratic Republic, the Federal Republic of Germany and Denmark.

● A new input into the Swedish nuclear energy debate promises to heat the simmering controversy yet again. This time the fuel is the publicity being given to a report prepared at Cornell University, New York; by Professor Robert O. Pohl, who challenges the traditional wisdom that nuclear energy causes fewer health problems than result from the same amount of electricity produced from coal. The report evidently asserts that the effects of radioactive waste from uranium mines should be included in calculations of nuclear health risks. On this basis, nuclear energy is said to be between 100 and 10,000 times as dangerous as has previously been thought.

Professor Pohl is reported to be critical of calculations done by the US Environmental Protection Agency (EPA) in a 1973 report *Environmental Analysis of the Uranium Fuel Cycle*. According to the EPA's estimates, the slag heap from a mine extracting uranium to produce about 1,400 million MWh of electricity would cause 60 cases of radiation damage—mainly lung cancer—after 100 years; 95% of these cases, it was estimated, would lead to death. So an annual rate of production of 10 million MWh would result in almost 0.4 deaths after 100 years. Professor Pohl points out that the slag at the mine contains thorium-230, which —by way of radium-226—decays to (radioactive) radon-222. During the decay of the thorium (and only 0.091% will have disappeared after 100 years) the slag will emit radon at a rate which Pohl estimates could result in a total of 396 deaths—presumably over more than 100,000 years. These calculations put nuclear energy in the same order of risk to health as coal-fired energy.

Scandinavian diary

from Wendy Barnaby, Stockholm

The report also questions the platitude that, although nuclear waste is dangerous, its volume is small enough to be controlled. On the contrary, if the slag is reckoned as part of the waste the disposal problem becomes enormous. For Sweden especially the difficulties would be immense: a ton of Swedish shale contains only 300 grams of uranium. The uranium required to produce 10 million MWh leaves 1 million tons of shale behind. Sweden's present annual electricity production is about 85 million MWh. The percentage of this provided by nuclear energy is negligible at the moment, but it is expected to rise to 12% by 1985.

Professor Pohl reportedly admits that thorium-230 and radon-222 would be formed wherever uranium occurs naturally, irrespective of whether it were mined or not. But he maintains that radon can escape more easily from the broken ground of a mine than from an undisturbed terrain. The effect of his views on the Swedish debate has yet to be seen; but it will probably provide another example of the fact that what may burst with a bang over the heads of the antinuclear lobby is, to the ears of the pronuclear government, merely a whimper.

● Experiments carried out by the Swedish Water and Air Pollution Laboratory show that the use of commercial preparations labelled 'non-toxic' and sold to disperse oil leaks in water has a far worse effect on marine life than the oil itself. The results indicate that if a dispersant is used to clean up oil spills, the effect on most marine life will be more severe and last for a longer time than if no dispersant is used.

The experiments, reported in the latest edition of the Royal Swedish Academy of Sciences magazine *Ambio*, were carried out on two dispersants commonly used throughout Europe: BP 1100X (British Petroleum) and Finasol OSR2 (Fina SA). According to the Swedish researchers, normal toxicity experiments are done with adult organisms under abnormal external conditions and for a short length of time On the grounds that such testing should be concentrated on the most vulnerable part of the organisms' life cycle, the Swedes used larvae instead. The type of larvae they worked with (Baltic herring, *Clupea harengus membras L.*) occurs naturally in the upper 10 metres of the water column, and is therefore especially susceptible to oil spills

The tests were done in jars containing brackish sea water to which was added Venezuelan crude oil with a density of 0.868 and a total sulphur percentage of 1.9. Some of the jars also had a dispersant mixed in. A control experiment with larvae in sea water only was carried out in parallel.

In the first set of experiments, in which larvae were exposed to different concentrations of water–oil and water–oil–dispersant mixtures, the larvae reacted 50 to 100 times more strongly to a mixture of dispersant and oil than to oil alone. The larvae deteriorated by swimming abnormally, then suffering injuries and finally dying.

In the second test, larvae were exposed to mixtures of different ages (0, 24 and 72 hours). The behaviour of the larvae showed that when oil and water were newly mixed, the resulting toxicity was higher than in older mixtures of oil and water. When a dispersant was added, however, the mixture's toxicity was even higher, and it remained almost unchanged after 24 and 72 hours.

news and views

Dynamics of chemical and biological reactions

from Henryk Eisenberg

IN a recent elegant study (Ben Basat and Bloomfield, *J. molec. Biol.,* **95,** 335; 1975) the kinetics of the spontaneous assembly of heads and tails of bacteriophage T4D, to form non-infective tail fibreless particles, were investigated. The authors used inelastic (or rather quasielastic) light scattering to follow an approximately 10% decrease in diffusion coefficients resulting from the attachment of the asymmetric tails to the icosahedral heads of the phage. The reaction was slow enough (the half time was about 500 s at the concentrations used) to be followed by measuring the average diffusion coefficients of the particles as a function of reaction time. The major finding was that the bimolecular rate of the reaction is about 1/500 that predicted by Smoluchovski in 1917 for a diffusion-controlled reaction between two spherical particles; the discrepancy can largely be explained by orientational factors. Careful analysis of the limits of and errors in quasielastic light scattering showed its usefulness as a tool in phage assembly studies, preferably in conjunction with other physical methods and with biological infectivity assays.

Fluctuations in time domain
Some readers may not be familiar with quasielastic light scattering. Note that one may associate with any process a 'noise' deriving from fluctuations around equilibrium states of particles in a system. Until recently it was not possible to determine the frequency spectrum of the fluctuations, nor to follow the evolution of this spectrum in time. From the time-averaged squares of the fluctuations classical elastic light scattering is derived. Fast electronic techniques and coherent monochromatic sources of radiation now make it possible to extract dynamic or kinetic parameters from the time-resolved analysis of the fluctuations.

The analysis is quite general and by no means restricted to the study of the fluctuations of scattered light. Fluctuations of fluorescence (Elson and Magde, *Biopolymers,* **13,** 1; 1974; Magde, Elson and Webb, *Biopolymers,* **13,** 29; 1974) or of electrical conductivity (Feher and Weissman, *Proc. natn. Acad. Sci. U.S.A.,* **70,** 870; 1973) in solutions, and also in nerve fibres, have been successfully examined. All that is required is that a change in the state of the system manifest itself by way of a measurable physical parameter. In technical terms the signal at a given time is compared (correlated) with itself at some previous time. In a subsequent averaging process the 'autocorrelation' function is formed. This is the Fourier transform of the spectrum and can be obtained by a simple mathematical operation. Those of us raised on the intuitive notion of the changing pitch of the whistle of an approaching or departing locomotive will easily recognise the Doppler spectrum—broadening due to the moving or reacting particle. It just so happens that modern technology often favours measurement in the time rather than the frequency domain (see *Photon Correlation and Light Beating Spectroscopy,* edit. by Cummins and Pike; Plenum, 1973; Chu, *Laser Light Scattering;* Academic, 1974; Berne and Pecora, *A. Rev. Phys. Chem.,* **25,** 233; 1974). A very broad range of phenomena has conveniently become accessible, in an experimental range having time constants corresponding to frequencies between 1 and 10^8 Hz.

In the study of the kinetics of macromolecular association and transconformation reaction two cases may be distinguished. The first case, which is also the topic of the Ben Basat and Bloomfield paper, focuses on the situation in which the reaction rate is slow compared to diffusion. If reactants and products have different translational diffusion coefficients, the scattered spectrum or autocorrelation function is that of a polydisperse system and can be resolved to provide concentrations of the various participants in the reaction. If the time of measurement is fast compared to the time of the reaction, the evolution of the latter is a function of time, and therefore the kinetic constants can be evaluated. This is what Ben Basat and Bloomfield have successfully done in the T4D phase head–tail assembly system.

The second case considers a reaction in dynamic equilibrium, in which the reaction rate is greater than, or comparable to, the diffusion rates for the reacting species. In this case theory predicts that the time autocorrelation function measured will be a sum of exponentials, whose decay constants are functions of both diffusion and reaction rates. However attractive this prediction may sound, conflicting and largely unsubstantiated claims have in the past resulted from studies of this kind. Decay curves of the autocorrelation function of reacting species are usually too complex to be resolved in a simple sum of exponentials in a unique, meaningful, way. But more recently Uzgiris and Golibersuch (*Phys. Rev. Lett.,* **32,** 37; 1974) reported a decay term independent of scattering angle in scattered-light intensity fluctuations from haemoglobin solutions. They attribute these fluctuations to the haemoglobin association–dissociation reaction; variations in experimental conditions affect the excess fluctuations in a predictable way.

Variations in method
Other examples exist, not necessarily related to the analysis of scattered light. In their very imaginative study, Feher and Weissman applied a constant voltage across an aqueous solution of an electrolyte, beryllium sulphate, in ionisation and hydration equilibrium. Fluctuations, on a fast time scale, occurred around the equilibria positions, and the reactants carried unequal charge. Therefore strong time-dependent fluctuations were observed in the ionic conductance and it was possible to measure the autocorrelation function of the conductance and derive meaningful kinetic constants for the ionisation process. These were in good agreement with results earlier obtained by the more perturbative temperature jump method. In another pioneering study Elson and his collaborators took advantage of the fact that the fluorescence of a dye, ethidium bromide, is strongly quenched upon binding to DNA. Once more fluctuations of binding (hence of fluorescence) occurred around the equilibrium state of the reaction. If a suitable exciting laser light was now focused on a tiny volume of the reac-

tion mixture, the concentration of fluorescing particles could be studied as a function of time and the autocorrelation function formed. The kinetic constants derived again were in good agreement with relaxation spectroscopy results.

In variety there is spice. New information can be derived from experiments in which external fields are imposed on a fluctuating system at thermal equilibrium. Ware and Flygare used electrophoretic scattering (*J. Coll. Interf. Sci.*, **39**, 670; 1972) in which charge-carrying scattering particles (in random Brownian motion) acquire and additional linear momentum by an applied d.c. electric field. In this situation the spectrum, or the autocorrelation function, can be clearly decomposed into contributions coming from the random, Brownian motion, and from the electrophoretic mobility of the particles. The resolution of the method is thus that, in priniciple, the motion of different particles, moving with different velocities, can be clearly distinguished. Hydrodynamic and sedimentation fields can, in principle, also be applied. In recent applications, Mustacich and Ware (*Phys. Rev. Lett.*, **33**, 617; 1974) reported on the study of protoplasmic streaming by laser light scattering and Josefowicz and Hallet (*Appl. Optics*, **14**, 740; 1975) described a sophisticated electrophoretic light scattering assembly suitable for analysis at low sample concentrations.

Clegg, Elson and Maxfield (*Biopolymers*, **14**, 883; 1975) introduced a new method in which a reaction is perturbed by repetitive application of a small pressure change. Reaction progress is followed by monitoring optical properties such as absorbance, fluorescence or light scattering. Phase-sensitive detection methods are used to enhance the low signal-to-noise ratio resulting from the slight perturbation. Yet the slight perturbation allows the analysis of closely spaced microstates, whereas the conventional perturbation methods, such as temperature-jump, often cause the system to transverse many intermediate conformations in the course of a single observation. This is of particular importance in reactions that involve the folding of biological macromolecules, in processes involving a multitude of partially ordered intermediate conformations.

In conclusion, a number of new tools for the study of reaction rates in chemical and biological reactions are being developed. In these new techniques advantage is taken of the spontaneous fluctuations around equilibrium states in the systems under study and, in other-instances, only minor perturbations are superimposed on these natural fluctuations.   □

Piezoelectric polyvinylidene fluoride

from Paul Calvert

THERE has recently been considerable interest in the development of piezoelectric (producing a voltage when stretched) polymer films. The current champion is polyvinylidene fluoride (PVDF (CH_2–CF_2)$_n$) which has a piezoelectric modulus d_{31} of 1–2×10^{-7} c.g.s.–e.s.u. compared to about 18×10^{-7} for barium titanate. The polymer, having the advantage of being available as a flexible film, is ready for use in microphones and possibly in loudspeakers. The origin of the piezoelectric effect in PVDF is still unknown and the matter is considerably confused by the complicated phase behaviour of this polymer. A pair of papers by Murayama and coworkers (*J. Polymer Sci.* Physics Edition, **13**, 929 and 946; 1975) of the Kureha Chemical Company in Japan, one of the two commercial producers of this polymer, go a long way towards clarifying the question if not to producing answers.

In 1969 Fukada (*Jap. J. appl. Phys.*, **8**, 960) and Kawai (*ibid.*, 975) showed that a piezoelectric effect could be produced in stretched and oriented films of several polymers when they were poled by applying an electric field of 500 kV cm^{-1} across the thickness of the film. When poled most polymers acquire a stable surface charge and are known as electrets. Except for PVDF there is no piezoelectric effect if the polymers are unoriented (Cohen and Edelman, *J. appl. Phys.*, **42**, 3072; 1971). This seems reasonable because the orientation process will tend to produce extended chain conformations of the polymer and in the vinyl polymers this would result in alignment of the permanent dipoles such as that of the –CF_2– group in PVDF. If the electric field caused these to point preferentially in one direction the material could be piezoelectric.

What makes PVDF special is its phase behaviour. There are three crystal forms: I, also known as β, II or α, and III, each of which can be produced by precipitation from a suitable solvent. Cooling from the melt gives form II which converts to form I on stretching. In form I the chains are extended so that the dipoles within one chain are parallel and the chains are packed into an orthorhombic lattice so that the dipoles on individual chains are parallel (Lando *et al.*, *J. Polymer Sci.*, A–1, **4**, 941; 1966) Obviously this form should be piezoelectric if it is properly oriented. Doll and Lando (*J. macromolec Sci. Phys.*, **2**, 205; 1968) showed that copolymerisation of tetra-

fluorethylene (CF_2=CF_2) with the vinylidene fluoride promoted formation of phase I and suggested that the 'head to head' groups (–$CH_2CF_2CF_2CH_2$) normally present at 5–10% in PVDF promote form I. Tadokoro *et al.* (*Macromolecules*, **8**, 158; 1975) go into this in more detail with infrared and Raman spectra and discuss the structures of form II, where the dipoles cancel, and form III which is similar to form I.

Hayakawa and Wada (*Adv. Polymer Sci.*, **11**, 1; 1973) have discussed the possible origins of piezoelectric effects in polymers and distinguish between intrinsic effects due to oriented dipoles and extrinsic effects due to trapped charges. On the face of it the former looks more likely for PVDF where the piezoelectric modulus increases with the content of form I whether produced by stretching or copolymerisation. Since the dipoles in form I are aligned it is simply necessary to assume that the crystals realign in the electric field or that more form I arises during the poling.

One observation that clashes with this is that of a negative piezoelectric modulus d_{33} in the film thickness direction by Burkard and Pfister (*J. appl. Phys.*, **45**, 3360; 1974). Any simple dipole model would predict a positive value; that is the film should get thicker when a field is applied parallel to the original poling field.

Murayama *et al.* report measurements on the decay of surface charge after poling. PVDF electrets have a charge of the same polarity as the field (homocharge) which is probably due to charges injected from the electrodes. This decays over a few hours to leave a charge of opposite sign (anomalous heterocharge) which is stable, even above the poling temperature. This anomalous heterocharge is correlated with, but not the origin of, the piezoelectric effect. They also observe that if a polyester film is placed between the positive electrode and the PVDF during poling the piezoelectric modulus and heterocharge are much reduced; between the negative electrode and the film it has little effect. No evidence is found for the production of more form I on poling and they argue that reorientation of existing crystals is very unlikely. Thus they conclude from their observations that the piezoelectric effect cannot be due to permanent dipoles but must be caused by the local migration and trapping of charges in the form I crystals. This process is somehow associated with charge injection from the positive electrode. The matter cannot really be considered settled until more is known about the nature of the induced dipoles and why form I in PVDF is particularly suitable By then there will probably be a new champion polymer.   □

Deuteron–gamma angular correlations

from P. E. Hodgson

WHEN deuterons are inelastically scattered by nuclei they leave the target nucleus in an excited state, which usually decays by gamma emission. The angular correlation between the inelastically scattered deuteron and the subsequent de-excitation gamma ray can provide important information about the mechanism of the reaction.

An illustration of this has recently been provided by the work of Scheib, Hofmann and Vogler (*Phys. Rev. Lett.*, 34, 1586; 1975) on the inelastic scattering of 10 MeV deuterons by ^{24}Mg. The angular correlation was measured for gamma rays emitted in the reaction plane, and in this case the correlation function is given by

$$W(\varphi_\gamma) = A + B \sin^2(\varphi_\gamma - \varphi_1) + \\ + C \sin^2 2(\varphi_\gamma - \varphi_2)$$

where φ_γ is the angle between the beam direction and the angle of emission of the gamma ray, and A, B, C, φ_1 and φ_2 are constants whose values are obtained by fitting the experimental data. The constants A, B, and C, are related to products of the reaction substate amplitudes, which may be calculated from a detailed theory of the reaction. This makes the correlation function much more sensitive to the details of the reaction than is the differential cross section, which is given by the sum of the absolute squares of all the reaction amplitudes. The sensitivity allows the correlation function to tell us more about the details of the reaction mechanism than we could learn from the differential cross section alone.

This is strikingly shown by the ^{24}Mg results. The figure shows the differential cross section compared with calculations using two different models of the reaction. The upper curves show the results of distorted wave calculations with three different sets of distorting potentials and assuming that the reaction takes place in a single step from the ground state to the 2+ excited state. The lower curves show similar calculations that also take into account the coupling to the 4+ state, so that some of the excited 2+ states are formed by first exciting the 4+ state directly, followed by de-excitation to the 2+ state. All the calculations used the symmetric rotator model including spin-orbit interactions.

When comparing such theories with the experimental data we look for a good fit to the main forward peak, which is given by both theories and all potentials, together with a qualitative agreement with the rest of the cross section. The angle and width of the second peak are given quite well but its magnitude is somewhat underestimated by all calculations, rather more so by the theory including the two-step

process than by the one-step theory. All these fits are acceptable, but do not distinguish between the two reaction mechanisms.

When we examine the deuteron–gamma correlation data the comparison is quite
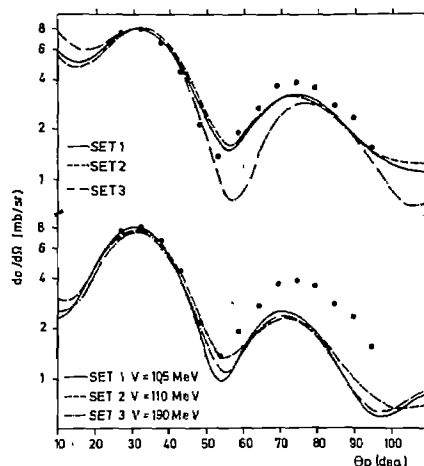
Differential cross section for the inelastic scattering of 10 MeV deuterons by ^{24}Mg with excitation of the lowest 2+ state compared with distorted wave calculations with three sets of distorting potentials using the one-step theory (upper curves) and the two-step theory (lower curves). V is the depth of the real part of the distorting potential.

different. If the results for the parameter C in the above expression for $W(\varphi_\gamma)$ are compared with the results of the six calculations already mentioned, the greater sensitivity of the correlation function is obvious. The three calculated curves for the one-step theory differ markedly and none fits the data, even qualitatively; however two of the calculations for the two-step theory are in good qualitative agreement with the data, and in particular give the deep minimum found around 55°. These two potentials are quite similar, and correspond to the deuteron potentials known to be physically realistic by calculations based on the nucleon–nucleus potential and using the folding model. The third potential for the two-step theory is physically unrealistic and also fails to give a good fit to the angular correlation data.

It is of course well known from many other studies that two-step processes must be taken into account in a detailed treatment of inelastic scattering, and this is confirmed by the correlation analysis. What is new is the striking way that it definitely excludes the one-step process as a sufficient explanation; this shows that the correlation analysis could be a powerful way of determining the reaction mechanism in cases where it is not already known. □

Tunable alkali halide lasers

from John Walker

TUNABLE lasers, particularly dye lasers, now cover the whole of the visible spectrum, and are currently revolutionising optical spectroscopy. Unfortunately dye lasers do not operate in the near infrared, a region of great importance in molecular spectroscopy, pollution detection and fibre optic communications. Hence the significance of a recent paper by Mollenauer and Olson (*J. appl. Phys.*, 46, 3109; 1975) who have demonstrated tunable laser action in potassium chloride, and have suggested that the wavelength range 0.9 to 3 μm could be covered by suitable choice of alkali halide. These compounds, so important in our understanding of solid state physics and of point defects, have at last found a practical application.

Laser action over the range 2 5 to 2.9 μm was achieved using the $F_A(II)$ centre in lithium-doped potassium chloride pumped by a krypton ion laser at 647.1 nm. An input power of 40 mW was necessary when the KCl crystal was at 77 K, and lasing could be induced at temperatures up to 200 K.

The operation of the laser can be explained as follows. The $F_A(II)$ centre consists of a halide vacancy (an F centre) trapped by an impurity

metal ion—in this case lithium occupying a potassium lattice site. The centre is excited by the pump laser and then relaxes by rearrangement of the chloride ions around the vacancy. A population inversion is thereby achieved between the relaxed excited state and the unrelaxed ground state, which is emptied by further atomic rearrangement into the relaxed ground state. The system is therefore an ideal four-level laser.

Similar arguments apply to the F and the $F_A(I)$ centres, but in their case the excited state relaxation is very small, and there are competing de-excitation processes, making them unsuitable for laser action. However, two other potentially useful defects are the F_2^+ centre (a pair of adjacent halide vacancies), and the $F_B(II)$ centre (similar to the $F_A(II)$, but with two impurity atoms). These centres should enable a total tuning range of 0.9 to 3 μm to be achieved, and Mollenauer and Olson are currently trying to construct an F_2^+ centre laser using KCl.

The combination of simplicity, tunability, low pump power and potential frequency stability makes alkali halide lasers a promising prospect in the near infrared.

HL-A, the major histocompatibility system of man, which has its analogue, *H-2*, in the mouse, and also in many other species, is now known to contain many genes controlling cell surface determinants, immune response differences, some components of the complement system (in man C3 proactivator (Bf), C2 and probably C4) and perhaps other related functions connected in general with cell–cell recognition. The *HL-A* system was first defined by a series of antigenic specificities—identified by serological techniques using peripheral blood lymphocytes—which were assigned to two separate series (LA and FOUR) corresponding to two closely linked loci, now called *A* and *B* respectively, with multiple alleles. Although matching' for transplantation using these antigens, which was the original impetus behind their study, has turned out to be more difficult and complex than was hoped, knowledge of the wide range of important functions controlled by genes in the region has grown from these original serological studies. The earliest data on the recombination fraction between the *A* and *B* loci already indicated that the *HL-A* region probably included very many genes, at least hundreds, if not a few thousand.

The development of the *HL-A* system has been greatly stimulated by a series of international collaborative workshops started by D. B. Amos of Duke University, North Carolina, in 1964. These workshops, which involved exchange of reagents among a large number of participating laboratories and the combined analysis of the resulting data, have each been major turning points in the development of knowledge of all aspects of the *HL-A* system. Thus, the second and third workshops organised by J. J. van Rood of the University of Leiden in 1965 and by R. Ceppellini of the University of Turin in 1967, placed the definition of the first described antigens on a firm footing and established that they all belonged to a single system of closely linked genes. The fourth and fifth, organised respectively by P. Terasaki of the University of California, Los Angeles in 1970, and by J. Dausset of the University of Paris in 1972, established that two linked loci control the main serological specificities and, through a world-wide series of population studies, the universality of this genetic model and the general distribution of the antigens among the various major human population groups.

The aims of the sixth workshop organised by F. Kissmeyer-Nielsen of the University of Aarhus, culminating in the meeting in Aarhus, were the definition of specificities identified by the mixed lymphocyte culture (MLC) test and the improved serological de-

Histocompatibility testing international

from W. F. Bodmer

The 6th International Workshop on Histocompatibility Testing was held in Aarhus, Denmark, on June 29–July 5.

finition of specificities of the more recently described *C* locus, as well as of newer specificities of the *A* and *B* loci. The MLC reaction is measured by the proliferative response when cultured together of lymphocytes from different individuals.

A WHO nomenclature committee, which met immediately after the workshop, recommended that with the increasing number of loci being defined in the region, *HL-A* should be the name given to the whole region, while the individual loci within it will be called *A* (formerly the LA or first locus), *B* (formerly the FOUR or second locus), *C* (formerly AJ or the third locus) and *D* for the locus controlling the determinants identified by MLC typing. The probable order of these four loci is *D,B,C,A*. New loci of the *HL-A* region will be similarly named as they become clearly identified.

MLC typing
Following the discovery by F. Bach (of the University of Wisconsin) and D. B. Amos in 1967 that at least the majority of MLC reactions were controlled by genetic differences in the *HL-A* region, further work by Amos and others established the fact that the determinants of this reaction were controlled by one or more loci that were genetically separable from *A* and *B*, although closely linked to them. Subsequently, a number of laboratories clearly established that it was possible to use MLC reactions to a set of specially chosen lymphocytes to define MLC determinants, namely to do 'MLC typing'. For this the one-way test is used (in which one of the two sources of lymphocytes to be mixed is inactivated, for example by X rays or mitomycin, so it can only act as a stimulus for the other, which is not inactivated) and stimulators are chosen from individuals known to be homozygous for the *HL-A* region, usually on the basis of family studies. Because of the high level of polymorphism for the *HL-A* system, such homozygous typing cells are most readily found amongst the offspring of parents who are related. If a test cell does not show an MLC response to a given homozygous typing cell, then it presumably has the determinant carried by the typing cell. Such reactions to a series of typing

cells can be analysed following the same principles used for the analysis o serological reactions, to establish speci ficities defined by similar reactions t a set of typing cells. For the workshop fifteen collaborating laboratories from the United States and Europe, guidec by E. Thorsby of the University o Oslo, each used the same 62 carefull; chosen representative homozygous typ ing cells to type a panel of up to 10(responders.

The resulting data, which undoubt edly represent the most extensive s far collected on MLC determinants were coded in a standard format b; each of the laboratories and analysec centrally by A. Piazza of the Universit; of Turin, with the help of L. Lamn and F. Jorgensen of the University o Aarhus. There are many statistica problems involved in the assessmen of MLC reactions and in the definitio of positive and negative response: which were clearly revealed by thi combined analysis. The availability o such a large body of data, howevei was a great help in the more or les clear cut definition of a series of eigh determinants of the *HL-A-D* locus which were seen to correspond t various determinants previously define by one or more different laboratories Each *D* locus specificity could be identi fied by a cluster of up to six differen typing cells which, for the most part reacted together when the correspond ing determinant was present on responder cell. Family and populatio data suggested that these six determin ants behaved as if controlled by series of alleles at the *D* locu; although it is clear that, as in the cas of the serologically defined specificitie of the *A*, *B* and *C* loci, new data ma lead to 'splitting' of these MLC de terminants into two or more sut specificities, and indeed to splitting o the *D* locus itself. A particular featur of the *D* locus determinants, whicl was clearly confirmed by the workshop was their tendency to be in strong link age disequilibrium with *B* locus allele: Linkage disequilibrium is the tendenc; for alleles at different loci, usuall closely linked, not to be associated a random in a population. It is in genera the main cause of population associa tions between functions controlled b linked genes, and so is the probabl basis for most of the known HL-A an disease associations which have bee: interpreted as due to linkage disequilit rium between alleles of the *B* locus an alleles at a linked immune respons locus. Thus, it is, for example, th DW2 specificity (formerly LD 7a; which is closely associated with th serologically defined specificity B (formerly HL-A7) and also very sign: ficantly increased in frequency amon patients with multiple sclerosis.

Serology

The serological part of the workshop was based, as before, on an exchange of carefully selected sera among the participating laboratories. Each of the 73 laboratories, including one or more from every continent (except Antarctica) typed up to 250 cells with the same set of 178 sera. The populations of cells included the laboratories' own well-typed panels, some families, some 'exotic' population groups and also, in the United Kingdom as a convenient source of B lymphocytes, lymphoblastoid cell lines and cells from patients with chronic lymphocytic leukaemia (CLL).

The combined analysis of this large body of data, which was organised by Julia Bodmer of Oxford University with the help of Piazza and others, first of all clearly establishes the definition of five specificities of the C locus. Specificities of this locus, whose existence was first suggested by L. Sandberg of the University of Goeteborg and E. Thorsby and their collaborators in 1970 and which was later confirmed by A. Svejgaard, University of Copenhagen, and others, have so far been more difficult to define than those of the better known A and B loci. This is, in large part, because alleles of the C locus tend to be in strong linkage disequilibrium with those of the B locus and so easily confused with them. Eighteen antigens of the A locus and twenty-four of the B locus were recognised by the workshop sera These included better definition of a number of previously suggested specificities, often 'splits' of already defined antigens This splitting of antigens is a common phenomenon in HL-A serology mainly as a result of the existence of cross-reacting families of antigens which are seldom at first separated by the available antisera.

Ia-type specificities

A most important byproduct of the workshop was the simultaneous description by at least nine different laboratories of various approaches to the serological identification of Ia-type specificities. In the mouse the genetic region between the H-2K and H-2D loci (which correspond to HL-A-B and HL-A-A respectively) has been shown to contain, in addition to genes controlling immune response, further genes controlling a new set of serological specificities called Ia for immune associated Unlike the K and D, or HL-A-A, B and C antigens, the Ia antigens have a tissue distribution that is relatively specific, including especially B, but excluding T lymphocytes. Similar antigens were first described in man by van Rood and coworkers using inhibition of the MLC reaction as a screen and then immunofluorescence of

peripheral blood B lymphocytes. Other approaches to the identification of human Ia-type sera include in particular the use of B-derived lymphoblastoid cell lines and CLL cells, as well as partially purified peripheral blood B lymphocytes and immunisation between unrelated individuals identical at the HL-A-A, B and C loci. Ia-type antibodies are quite commonly found in the usual HL-A-A, B and C locus typing sera (which are mostly obtained from multiparous women who make these antibodies as a result of foetal–maternal stimulation) and were shown by W F. Bodmer of the University of Oxford and H. Dick of the Glasgow

Royal Infirmary and their collaborators to be present in just under one-third of the workshop sera. Several reports indicated, as might be expected, close association between Ia-type serological reactions and MLC determinants of the D locus. Clearly, serological Ia typing is much easier than MLC typing and so may become a powerful tool in disease association and other related studies. There is also the definite possibility that Ia specificities will be of greater direct importance for the problem of clinical transplantation than are the specificities of the HL-A-A, B and C loci.

This workshop was as exciting and

Hydrous minerals in meteorites

from David W. Hughes

THE water content of rocks, whether they be terrestrial or meteoritic, can provide an important clue to their place of origin. Meteoritic minerals are nearly always fresh and undecomposed —which contrasts with many of the water-containing products of weathering and erosion which occur in such profusion on Earth. By studying the chemical composition and paragenesis (the groupings of different types of minerals) of meteorites it is found that in the main they must have crystallised from a fiery melt which had an exceptionally low water content in contrast to the wet volcanic terrestrial melts which solidify to form igneous rocks. Only in type 1 and 2 carbonaceous chondrites is water reasonably abundant. These were formed at low temperatures and have not been subsequently heated above 350 °C. They are thought to have compositions very close to that of the primordal dust which coalesced to form the Solar System and they contain many primary volatile components. These meteorites are thought to be parental to other types which are formed by complicated thermal evolution processes and geochemical depletion reactions.

Ashworth and Hutchison in this issue of Nature (page 714) report having found hydrous minerals in the meteorite Nakhla, a diopside–olivine achondrite which fell in Egypt in 1911 and in the meteorite Weston (a chondrite which fell near Weston, Connecticut in 1807). Both are meteoritic types in which water is rare.

The water in Nakhla and Weston is in the form of iddingsite, a mixture of hydrothermal alteration products of olivine ($(Mg, Fe)_2 SiO_4$). The iddingsite is found as red-brown veins along cracks in the olivine. These veins were probably formed by shock deformation, and water present at this time perco-

lated down the cracks and caused mineral alteration.

Ashworth and Hutchison conclude that magmatic water must have been present during the formation of this shocked olivine, and that the meteoritic mineral originated on a body in the Solar System that had a hydrous atmosphere. There is however still the possibility that the meteorites were 'dry' when they left the parent body and that they picked up the water later. The water could also have come from an admixture of some carbonaceous chondritic material in with the meteorite; this however has not been chemically identified. The interaction between solar wind ions and meteoritic minerals causing hydrogen implantation is discounted because lunar breccias, which have been exposed to the solar wind for aeons, are generally anhydrous. That leaves terrestrial weathering as the alternative source of water. Was the hydrous mineral produced by the hydrolysis of primary lawrencite ($FeCl_2$) by water vapour in the terrestrial atmosphere? As Weston contains an order of magnitude more water than Apollo 16 rocks this requires a very high initial content of chlorine. Both Nakhla and Weston were observed falls and were collected soon after hitting the Earth. However as Nininger (Out of the Sky, Dover, 1952) points out, we never see a truly unweathered meteorite, the atmosphere through which they fall being nearly saturated with water—to say nothing of the air in the vicinity of the museum shelf on which they have been sitting for over half a century.

So the mystery remains—did the meteorites come from a wet planet or have they picked up their water later? It would be heartening to think that Earth has not been the only wet place in the life of the Solar System.

stimulating as its predecessors, reflecting the enormous effort devoted by Kissmeyer-Nielsen and his associates to its organisation and showing once again the value of such world-wide collaborative studies. The next workshop will be organised in Oxford in about 2 years' time and will have as its main theme the definition of human Ia specificities, their relation to MLC determinants and their significance for disease association and clinical transplantation. ☐

Reliability of molecular phylogenetic trees

from David R. Thatcher

IN principle a comparison of the primary structure of the same protein from a number of related organisms should provide evolutionary information of high precision. The amino acid sequence of a protein is a translated copy of the sequence of nucleotide bases in the structural gene and therefore is the one phenotypic character that is a direct reflection of the structure of the genome. The comparative morphology of amino acid sequences should therefore permit the deduction of some of the discrete mutational steps which have occurred during the evolution of an organism. Providing no genetic transfer has occurred and the gene has retained the same function throughout its history, a phylogenetic topology derived from amino acid sequence comparisons should represent the true ancestral relationships of the organisms expressing that particular gene. Molecular phylogenetic methods have the potential advantage of being able to distinguish evolutionary relationships where the classical methods of evolutionary biology do not produce a convincing answer, for example where the fossil evidence is controversial or absent or where there is little morphological diversity to compare.

In practice little new phylogenetic information has been derived from the construction of trees based on molecular evolutionary data and the method has received a somewhat hostile reception from other evolutionary biologists (these points are fully discussed in the excellent review of J. Williams in *The Chemistry of Macromolecules: Proteins*; Medical and Technical Publishers, 1974). The most damaging criticism proposed is that data based on a single structural gene cannot possibly reflect the evolution of the whole genome. Nevertheless, trees constructed by comparing sequences of vertebrate cyto-

chromes *c*, haemoglobins and fibrinopeptides are each more or less in agreement with the accepted phylogeny for this group, showing that the method at least works even if its precision is not as great as at first hoped.

The resolution of the sequence method is unquestionably restricted and if future projects are going to be attempted with an eye to solving particular phylogenetic problems, these limitations must be fully defined. These limitations are due to two main factors. First, to what extent is the assumption of parsimony justified at the molecular level? There is no absolute way of knowing the degree to which parallel, back or convergent mutations affect observed amino acid similarities. Second, if one accepts the most parsimonious solution as being the actual evolutionary history of a gene, the numerical problems involved in calculating this solution for even a small number of alternative topologies is formidable.

Peacocke and Boulter (*J. molec. Biol.*, **95**, 513; 1975) have begun to assess the extent to which these technical limitations have affected their conclusions on higher plant evolution. The evolution of a protein was simulated in a computer and the reconstruction of this hypothetical phylogeny was attempted using two different methods. The accuracy of each procedure was then estimated by determining the number of times a branch had to be moved in order to restore the original topology. The two methods employed were the ancestral sequence method of Dayhof (*Atlas of Protein Sequence and Structure*, 1972) and the matrix method of Moore, Goodman and Barnabas (*J. theor. Biol.*, **38**, 423–457; 1973). The ancestral sequence method infers the structure of nodal ancestral sequences as it generates a tree by adding one sequence at a time to the topology. New branches are added with respect to the most parsimonious sequences (real and ancestral) already in the tree. In the matrix method a distance matrix is calculated and an approximate tree is produced by successive pairwise clustering. This tree is then adjusted until hypothetical mutational distances (computed from the tree by comparing the mutational distances of two neighbours with the average mutational distance of all other members of the tree) approach the actual values of the original distance matrix. Applied to the model data the accuracy of the ancestral sequence method became progressively worse as the number of observed substitutions in the data set was increased. In contrast the matrix method, although less accurate than the ancestral sequence method at lower degrees of substitution, improved as the

variation between the sequences increased. Phylogenetic trees were then constructed from model data which varied to the same extent as the real data Boulter's group has accumulated on higher plant cytochrome *c*. The error observed in tree building with the model data and the ancestral sequence method ranged between 2% and 8% whereas the matrix method operated with an error of 9% to 15%. For a rapidly evolving protein like plastocyanin methods were equally successful in predicting the correct topology (error 8% to 12%).

Before molecular phylogenies are considered seriously by systematicists, some attempt must be made to quantify the errors inherent in each method; from the extent to which tree topologies may be influenced by sequencing mistakes to the probability of a given tree being the most parsimonious solution possible. Peacocke and Boulter have shown that an optimal amount of amino acid variation is required for the most accurate topological assignments by the ancestral sequence method. As structural genes have characteristic rates of evolution, which vary greatly from gene to gene, only a limited range of accurate phylogenetic information can be obtained from the study of a single gene.

Convincing evidence is obtainable but only by investigating the best gene for a particular phylogenetic problem and applying the numerical method most suited to the data. ☐

Consequences of hydrothermal circulation

from Peter J. Smith

LAST year, Lister (*Eos*, **55**, 740; 1974) made a strong plea for the testing of the hydrothermal circulation hypothesis using deep sea boreholes near oceanic ridges. His argument was that there is growing evidence that certain observations, notably in the fields of oceanic heat flow and ocean floor mineralisation, can only be explained in terms of the penetration of water to substantial depths (possibly several kilometres) in the new crust forming at ridge crests.

Presumably it is still too soon to expect any results to have emerged from such a proposal. In the meantime, however, indirect support for hydrothermal circulation continues to accumulate. Lee and Von Herzen (*Geophys. Res. Lett.*, **2**, 201; 1975), for example, have now reported 15 new heat flow measurements from the South Atlantic triple junction near 55 °S, 0 °E —the zone in which the boundaries of the African, Antarctic and South

American plates meet. Although the data are few, they show a distinct geographical correlation; on ridge crests flanking axial valleys the heat flow is high, in the depressions of fracture zones and axial valleys it is intermediate to low and on ridge flanks it is low. At one extreme, four values higher than 4.0 μcalorie $cm^{-2} s^{-1}$ were observed on the crests of three ridge segments, whereas at the other, a value of 0.4 μcalorie $cm^{-2} s^{-1}$ was found in a depression of an axial valley near its intersection with an adjacent fracture zone.

The problem with these and previous similar results from less complex areas is that they conflict with the conditions of steady state heat conduction, by which heat flow should be higher through depressions and valleys than through ridges. Variations in the thermal conductivity of the sediments in which the heat flow was measured would be one possible way of reconciling observation with conduction theory, but in the case of the South Atlantic triple junction such variations do not exceed 50% whereas heat flow values vary by at least an order of magnitude.

Lee and Von Herzen thus favour an explanation involving hydrothermal circulation in the cracks produced by thermal and/or tectonic tensional stresses near the spreading axis. According to this view, which is apparently the only one offering any hope at present, the high heat flow over a ridge crest reflects the topographically controlled ascending limb of the circulation system in the permeable basement rocks (although it could result from conductive cooling of the crust following the sealing of cracks generated at the spreading centre). The intermediate to low values in the axial valleys may then be attributed to the circulation's descending limbs. Similar values in fracture zone depressions are not quite so easily explained in detail, although reasons for cracking and subsequent hydrothermal circulation there are not difficult to envisage.

The only real difficulty is in accounting for the low heat flow over ridge flanks. One possibility is that the irregular topography here supports a locally controlled hydrothermal system which imitates the wider pattern. Thus hot water would ascend through elevated terrain and descend in depressed regions. The observed low heat flow would then result from a known sampling bias towards local sediment-filled topographic lows. A second possible explanation is that secondary circulation occurs in cracks reopened during the dehydration of rocks previously hydrated near the spreading axes. But either mechanism affirms the crucial role of hydrothermal circulation.

A similar conclusion has also been reached by Piper *et al.* (*Earth planet. Sci. Lett.*, **26**, 114; 1975) but in a quite different context. Piper and his colleagues have analysed (for major, trace and rare earth elements and uranium isotopes) an iron-rich (about 30%) deposit dredged from the upper flank of Dellwood Seamount in the north-east Pacific. What attracted them to the sample in the first place was its iron-richness, because oceanic deposits with high iron contents (20–35%) and high Fe/Mn ratios (3–75) have previously been associated with active ridge spreading. Indeed, in overall composition and mineralogy the new sample closely resembles other iron-rich deposits attributed to volcanic hydrothermal activity. But do the separate elemental compositions and distributions also support such an origin?

As Dellwood Seamount is close to the North American continent, an obvious possibility is that some of the elements in the sample are terrigenous, introduced into the oceans by rivers. This view may be rejected, however, partly because the major element composition differs from that of metal deposits from hemipelagic environments and partly because the Th content and Th/U ratio are much lower than those of typical river muds. Are, then, any of the elements derived from sea water? The fact that the rare earth element pattern and the $^{234}U/^{238}U$ ratio differ significantly from those of normal sea water suggests that this is unlikely, at least for the rare earths and uranium

isotopes (and probably for others too). Indeed, although the absolute concentrations of rare earth elements are much lower than those in oceanic sediments and basalts, the pattern of these elements is almost identical to that of ridge basalt.

Taken together, these observations can only be explained on the assumption that the unusual composition of the iron-rich deposit derives from a suboceanic source. And the only known way in which this source may be tapped is by hydrothermal circulation which leaches out the metals from flow interiors and transports them to the surface. Admittedly there is a problem. The $^{234}U/^{238}U$ ratio is not only much higher than that of normal sea water, it is also much higher than that generally found in iron deposits now forming near known active volcanic centres. In the case of the Dellwood deposit it is therefore also necessary to assume that ^{234}U is leached from the rock preferentially. The point is, however, that even this anomaly can be accommodated within a hydrothermal system. ☐

Constructing semisynthetic polypeptides

from D. G. Smyth

A SEMISYNTHESIS is one that utilises a fragment of a natural substance as a prefabricated unit in the construction of a larger molecule. The term does not include the preparation of simple derivatives of a natural protein nor of course does it cover the chemical synthesis of peptides from their building bricks, the constituent amino acids. The hallmark of a semisynthesis is that it involves the combination of two components, the one a fragment of a natural substance, the other a product formed by chemical synthesis. This description would include the preparation of ribonuclease S by association of the subtilisin-produced S-protein (residues 21–124 of ribonuclease) with a synthetic S-peptide (corresponding to residues 1–20). The combination of natural insulin A-chain with synthetic B-chain, or *vice versa*, would also qualify as a semisynthesis, as would the conversion of porcine insulin to human insulin by replacement of the B-chain octapeptide.

In the field of peptide synthesis, the idea that an intermediate fragment might be obtained by excision from a protein of known structure is attractive. Such fragments can be obtained in pure form and without racemisation by the action of proteolytic enzymes;

A hundred years ago

IN the Paris International Maritime Exhibition there is a small object deserving of notice. It is a platinum wire placed in a bottle and ignited by electricity from a bichromate battery. It is intended to be immersed in the sea, and the light emanating from it is said to attract an immense number of fishes. Experiments have been tried lately on the coast of the Côtes du Nord department with a fishing-boat, and have proved very satisfactory, on a bank of sardines. The glass must be green or black, otherwise the fish are frightened by the glare and do not follow the submarine light.

from *Nature*, **12**, 388, Sept. 2, 1875

but difficulties arise in activating the fragment for further use. This is illustrated in a semisynthesis of human β-MSH by Burton and Lande (*J. Am. chem. Soc.*, **92**, 3746; 1970), utilising the porcine hormone as an intermediate. Human β-MSH possesses four more amino acids than porcine β-MSH and a tetrapeptide was therefore synthesised with the intention of extending the NH$_2$-terminus of the porcine molecule. Unfortunately porcine β-MSH has two NH$_2$ groups, the α-NH$_2$ group at position 1 and the ε-NH$_2$ group at position 6, and the coupling reaction led to the formation of products with the tetrapeptide attached at more than one site. Inevitably the yield of the required α-linked hormone was low. In semisynthesis it is clearly important that, before coupling, all reactive side chains should be protected to ensure that only terminal residues are free to participate in the formation of peptide bonds. Herein lies the difficulty.

Offord (*Nature*, **221**, 37; 1969) has reported that carboxyl groups in peptides can be protected under mild conditions by reaction with phenyldiazomethane. This forms benzyl esters at the side chains of all aspartic and glutamic acid residues but it also esterifies the C-terminal carboxyl group; the latter, however, can be regenerated selectively by the action of an esterase. When the fragment is initially obtained from a natural polypeptide by the action of trypsin, the same enzyme can be used to remove the C-terminal benzyl group. Similarly NH$_2$ groups can be reversibly blocked in a precursor polypeptide before the required fragment is released by proteolytic cleavage. Provided the peptide was initially present within the sequence of the precursor and not at the NH$_2$-terminus, the enzymically released fragment will have a free NH$_2$-group at the N-terminus; the ε-NH$_2$ groups remain protected. By these reactions, one fragment can be prepared with only a C-terminal carboxyl group and another with only an N-terminal NH$_2$ group, appropriate to the coupling reaction. In this way, linking of the first peptide to the second can be made to take place specifically between the C-terminus of the one and the N-terminus of the other. While these reactions seem straightforward, it is true to say that few reports have appeared in the literature on the preparation of new polypeptides by the combination of natural fragments.

A semisynthesis of a different type has involved the preparation of an insulin in which the B1 phenylalanine was replaced by iodophenylalanine, with retention of biological activity (Offord, *Nature*, **227**, 718; 1970). To perform the conversion, the A1 and B29 NH$_2$ groups were protected by

trifluoroacetylation and the remaining NH$_2$ group at B1 was blocked by reaction with phenylisothiocyanate. The B1 phenylalanine residue was removed by treatment with trifluoracetic acid, giving rise to a fragment of insulin which lacked only the B1 residue. Coupling of iodophenylalanine to the insulin fragment was easily achieved. Obviously the semisynthesis of this homogeneous derivative is more practicable than a total synthesis of the specifically iodinated insulin from the constituent amino acids.

Dyckes, Creighton and Sheppard (*Nature*, **247**, 202; 1974) have described an intriguing semisynthesis of an analogue of trypsin inhibitor. The 58-residue polypeptide was cleaved specifically at a methionylarginine residue by cyanogen bromide, which converted the methionine residue to homoserine lactone and conferred weak acylating properties on the fragment. A peptide bond then formed spontaneously between the homoserine residue and the adjacent arginine residue, restoring

the intact polypeptide chain. The product, which carried a residue of homoserine in place of methionine, retained the inhibitory action of the natural molecule. Corradin and Harbury (*Biochem. biophys. Res. Commun.*, **61**, 1400, 1974) performed a similar experiment with cytochrome *c*, which has no stabilising disulphide bridges. They found that cyanogen bromide cleavage gave rise to two fragments which could reunite; again the homoserine analogue retained biological activity.

In the general case, cleavage of a polypeptide at methionine would not be accompanied by a tendency for the fragments to recombine. Only in situations where the three-dimensional structure of the cleaved molecule is the same or very similar to that of the natural molecule will the newly released termini be held in juxtaposition for intramolecular condensation.

These are early days in the development of semisynthetic procedures. Future applications will be awaited with interest. □

Between molecules and collision complexes

from our Chemical Physics Correspondent

IT is almost three years (*Nature*, **240**, 257; 1972) since I drew attention to the identification of (HF)$_2$ and expressed the hope that H$_2$OHF would also be identified in the gas phase. This has now been done by Bevan, Legon, Millen and Rogers (*J. Chem Soc. Commun.*, page 341; 1975) who find a clear-cut microwave spectrum near 14 GHz and 29 GHz.

It is the hydrogen of the HF which forms the hydrogen bond; the O .... F distance is 268 pm and the dipole moment is 12.8×10^{-30} C m (3 82 debye). There is an intensity alternation associated with the spin statistics of the two equivalent H attached to the oxygen, but it is not clear if the whole molecule is planar (point group C$_{2v}$) or whether the fluorine lies out of the H$_2$O plane (point group C$_s$). As expected the intensity seems to be proportional to the product of the partial pressures of each component and with an optimum total pressure of 100 Pa the lines are necessarily broad and such clear identification must have required the use of much experimental skill.

But though hydrogen bonds have been known for some time and such gas phase molecules have long been postulated, the extension of this work to the charge transfer species (CH$_3$)$_3$ N.ICF$_3$ is even more remark-

able (Legon, Millen and Rogers, *J. Chem. Soc. Chem. Commun.*, page 580; 1975) even though it is based on earlier infrared evidence (Mishra and Pullin, *Aust. J. Chem.*, **24**, 2493; 1971). For this species, which is a symmetric top, lines for *J* from 30 to 37 span 26 GHz to 33 GHz and are identified. With only one value of *B* for such a large molecule the structural information is not very precise, but suggests an N .... I distance of 293.2 pm. Habit sets the expression 'N .:.. I' with dots suggesting a non-bonded distance; but really the charge transfer complex has now a clear-cut identity and perhaps a full bond, based on charge transfer, should be shown.

And also unexpected is the spectroscopic detection, this time with molecular beam apparatus, by Harris, Janda, Novick and Klemperer (*J Chem. Phys.*, **63**, 881; 1975), of the rare gas species Ar.OCS. Both radiofrequency and microwave transitions were seen and they establish the structure as basically T-shaped with the argon adjacent to the carbon at a distance of 358 pm, slightly greater than the van der Waals' radius sum.

All of these molecules with self-respecting structures, lifetimes and dipole moments fill in the gap between true molecules and sticky collision complexes.

review article

Ion implantation

G. Dearnaley*

Ion implantation has proved an elegant and successful technique, and in the manufacture of silicon devices, is now being extended both for research and practical applications in other materials, such as metals.

ION implantation is the process whereby controlled amounts of chosen foreign species can be introduced into the near-surface regions of a material in the form of an accelerated beam of ions. The energies used normally lie between 10 keV and 500 keV, with corresponding penetrations ranging from 100 Å to 1 μm, depending also on the target material.

The technique has proved highly successful for the fabrication of semiconductor devices, in spite of being in competition with well established processes of thermal diffusion or epitaxy, and requiring a relatively expensive accelerator and vacuum equipment. Some 200 accelerators are now in operation throughout the world and this number is expected to rise 10-fold—far in excess of the number used for nuclear research.

There are several reasons for the success of ion implantation. Among the most important are the control and resolution offered by this technique. Both the total amount and the purity of the implanted material can be accurately controlled and monitored, in a way that is impossible, for example, with diffusion, in which surface phenomena determine the dose which diffuses in. Furthermore, the concentration of impurities as a function of depth can be controlled by means of the ion energy, so that it is feasible to implant a buried layer of dopant. But for practical purposes, a crucial feature of the technique is its compatibility with photolithographic masking. This means that the well directed beam entering the surface produces a doped region which can be given a very high lateral resolution using conventional masking techniques (the etching of apertures through oxide films by use of a photosensitive resin overlayer).

The process also avoids certain problems associated with other techniques. Implantation, and the subsequent annealing of damage, are completed quickly and at relatively low temperatures, avoiding the difficulties associated with lengthy exposure to high temperatures; it is also very clean, being carried out in a vacuum, and could in principle be highly automated.

Its versatility is a further advantage, for a single accelerator can be used for many doping and other processes in device manufacture, with a schedule that is easily varied in such a way as to aid product development or the production of short runs of specialised devices. In materials other than semiconductors, the advantage lies in the possibility of introducing essentially any required species and producing a treated layer which is integral with the substrate.

Physical processes

When ions enter a solid, they lose energy through elastic collisions and through electron excitations. A reasonably accurate theory of the process was developed by Lindhard,

*Address: Atomic Energy Research Establishment, Harwell, Oxfordshire OX11 0RA, UK.

Scharff and Schiøtt[1], and this is widely used to calculate ion ranges and range distributions. Some results for silicon are shown in Fig. 1, from which it can be seen that the ranges of commonly used dopants (boron, phosphorus) are compatible with the dimensions of present-day microelectronic components (100–1,000 nm) with ion energies of a few hundred keV. Since the semiconductors are crystalline, the phenomenon of ion channelling[2] introduces some difficulties: this is the enhanced penetration of ions travelling along open crystallographic directions, as a result of small-angle collisions with the atomic rows. We have been able to show that this is the cause of a "tail" which extends beyond the expected maximum range (Fig. 2) and various techniques have had to be developed to cope with this.

Most semiconductors are covalently-bonded materials, with rigidly defined bond lengths and bond angles. Under ion bombardment this structure is vulnerable to disordering and in most cases it is necessary to restore crystallinity by thermal annealing at 800–1,200 K for about 20 min. Otherwise, electrically-active defects will dominate the effects of the atoms introduced. Figure 3 shows a transmission electron micrograph of a wafer of phosphorus-implanted silicon after annealing at 700 °C. There is a large amount of localised strain visible as dark regions, but the superimposed electron diffraction pattern shows that between these the crystal is relatively perfect. Fortunately, the electron transport properties of the semiconductor are also very good and the carrier mobility in the implanted zone may approach that of bulk material at the same impurity concentration. Moreover, the carrier density will (in silicon) correspond to the number of phosphorus or arsenic atoms introduced, although for boron the degree of electrical activity is commonly less than 100% because of the formation of complexes such as SiB_6. In some applications, the electrical effects of defects induced by bombardment can be put to good use. Proton bombardment will raise the resistivity of GaAs by seven orders of magnitude[3], and is increasingly adopted for producing the isolation between neighbouring devices. The physical mechanisms involved are only just becoming clear: once again technology has provided a spur to physics. Another important process is that of causing unwanted metallic impurities, such as Au, Cu, Ni, to diffuse to defects introduced by ion bombardment[4]. This is the technique known as "gettering", important for achieving reproducible device performance and low diode leakage currents.

Ion-implanted silicon devices

We can now consider the various semiconductor devices to which ion implantation has successfully been applied, illustrating the advantages I have mentioned. The first important structures

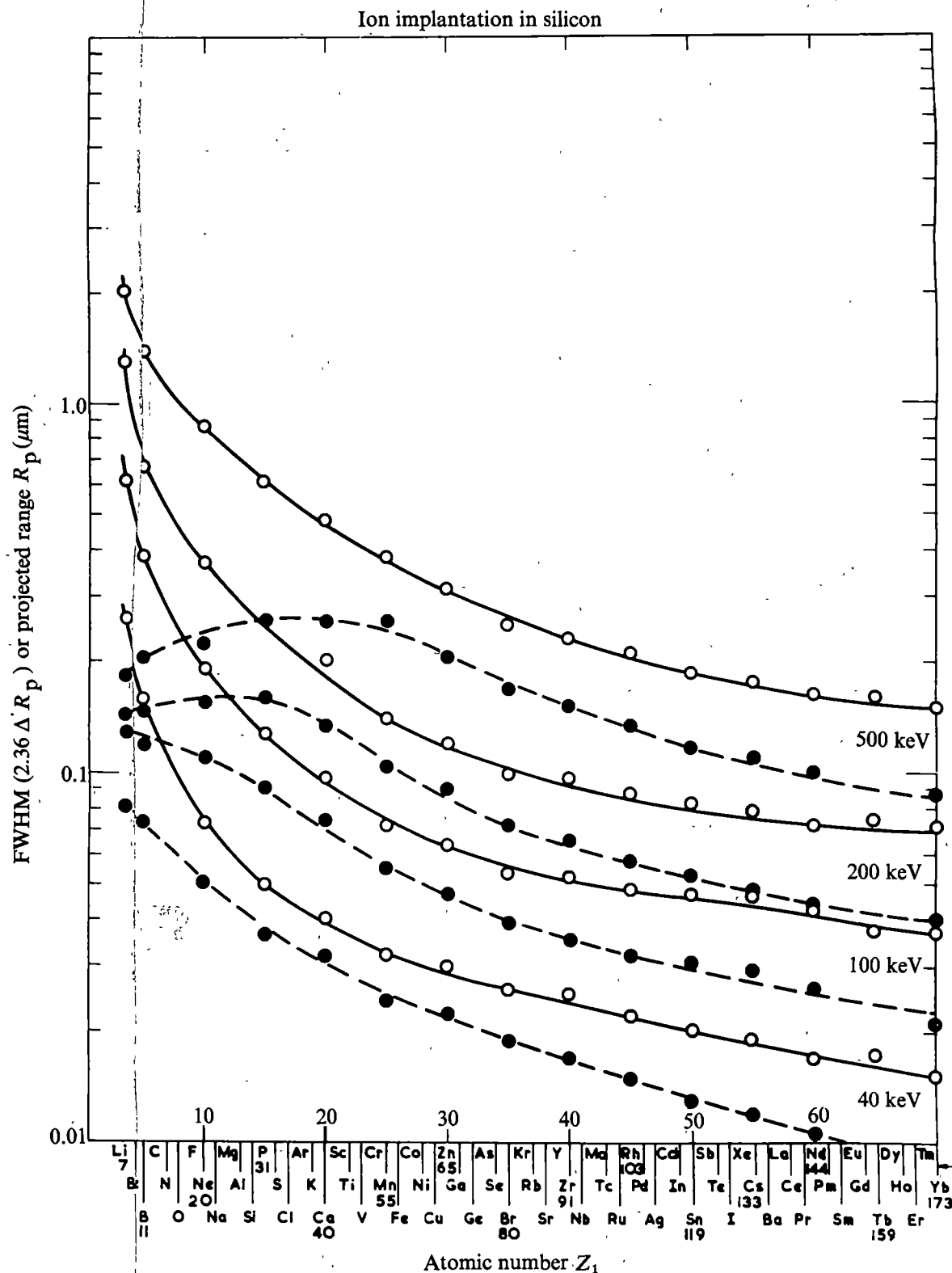## Ion implantation in silicon

Fig. 1 Ion ranges in silicon, as a function of atomic number, and the full-width at half maximum (FWHM) of the depth distribution, assumed to be Gaussian. ○, Projected range; ●, FWHM (projected).
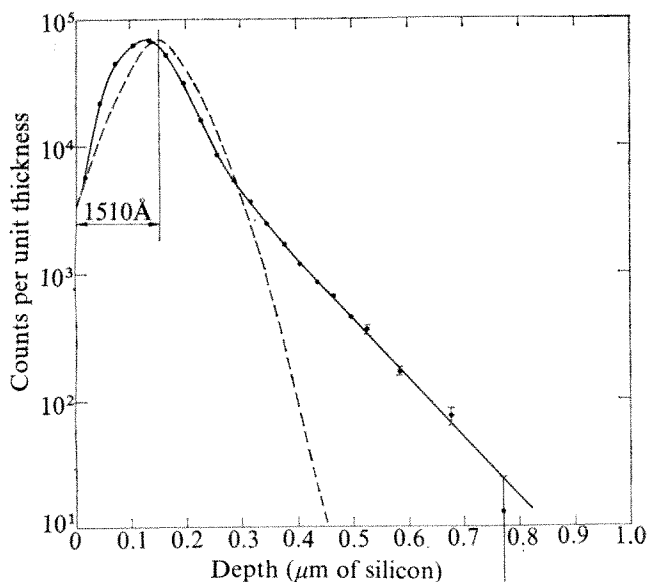
to benefit dramatically from the technique were the metal-oxide-semiconductor (MOS) transistors. In these the conduction takes place at the surface of a silicon crystal, and is controlled by the voltage applied to a metal "gate" electrode, electrically insulated from the silicon beneath by a thin layer of thermally-grown SiO_2. Difficulties in aligning the elements of this structure were overcome by using the metal gate as a mask for implantation. Excellent registration of the "source" and "drain" (input and output) regions of the transistor was

automatically achieved with a consequent reduction in stray capacitance. Next, the surface conducting region was doped by ion implantation to the required value, while the material surrounding the transistor was maintained at a higher resistivity. By this means parasitic capacitances that load the input and output were significantly reduced. Finally, it was shown that the conducting "channel" could readily be buried so that electrons or holes would travel in good crystalline material rather than at the silicon–oxide interface where lattice defects

would interrupt their motion. These factors allowed the dimensions of MOS transistors to be reduced and their speed of operation to be dramatically improved.
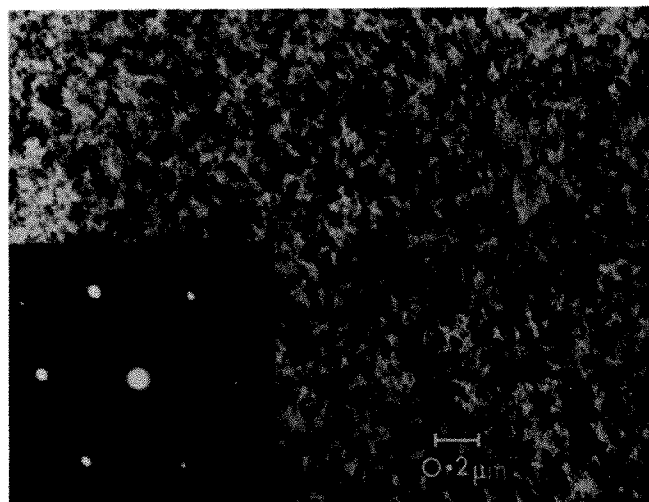
Integrated circuits, sometimes containing many thousand individual transistors on a single square of silicon, have become extremely important for electronic systems ranging from pocket calculators to military defence installations, and they were stimulated intensively during the development of space satellites. Besides the techniques described above, two other applications of ion implantation have been found which proved even more beneficial. The threshold voltage, at which conduction of an MOS transistor commences, is governed by impurities in the oxide layer or at the oxide–silicon interface. Bombardment with low doses of ions (not necessarily group III or group V dopants) alters the threshold voltage[5] in a remarkably controllable manner (Fig. 4). Secondly, it is desirable to construct the two main types of MOS transistor (called "enhancement" and "depletion" mode devices) within the same circuit, so that one can act as a load for the other. The result is a considerable saving in power consumption, important in pocket calculators or electronic wristwatches, and so on. The necessary doping, to produce p-type regions, or "wells",

Fig. 3 Transmission electron micrograph of an ion-implanted silicon crystal, after thermal annealing, together with (inset) an electron diffraction pattern from the same region. The former shows a dense network of defects, while the latter reveals that the intervening matrix is crystalline.

however, that charge be transferred efficiently from one element to the next, and here the "buried channel" produced by ion implantation has proved highly effective, removing the charges from the region containing traps at the oxide interface. It seems likely that ion bombardment gettering will be useful for removing metallic impurities which trap mobile carriers.

Bipolar transistors, in which the active elements (emitter, base and collector) are buried within a silicon crystal, still account for the major part of the device market. At first it seemed that they would not benefit from ion implantation, since the performance of implanted transistors was not reproducible. It is now realised that this was due, in part, to the effects of the "tails" on implanted ion distributions (Fig. 2), which vary in extent from one crystal to the next and which influence the depth of emitter-base junctions. It was found in the USA (R. J. Scavuzzo, R. S. Payne, K. H. Olson, J. M. Nacci and R. A. Moline, unpublished paper presented at the IEEE Electronic Devices Meeting, Washington DC, 1972) that if arsenic ions are implanted to form the emitter a subsequent "drive-in" diffusion will yield a desirable concentration profile

Fig. 2 Penetrating "tail" observed in the distribution of radioactive phosphorus ions implanted into a silicon crystal in a direction well removed from major channelling axes or planes. This tail is due to scattering into channelling directions. ● $\gamma 13$–X. $7°$ to $\langle 11 \rangle$. 120 keV. 5×10^{12} cm^{-2}. ^{32}P. Room temperature. (– – – –) calculated from Lindhard et al.[1].

in n-type silicon for these circuits is best carried out by implantation. In the most recent development both enhancement and depletion mode devices are implanted, and computer memories with almost 19,000 transistors on a single silicon "chip" have been produced. A microprocessor with the general capability of a PDP-11 computer is expected to be introduced this year, packed on to a chip which is comparable in size with the first calculator microcircuits.

Even these achievements are overshadowed by the rapidly developing "charge-coupled devices" in which, by an extension of MOS technology, arrays of metal–oxide–silicon capacitors are used to store charge which can be transferred to the adjoining storage element, just as in a solid-state analogue of the obsolete "Dekatron" glow-discharge indicator tubes. The charges can be liberated optically, in which case the device acts as a scannable photo-detector array, already on the market in the form of hand-held TV cameras. Alternatively, the devices can be used to store and process analogue information. It is important,
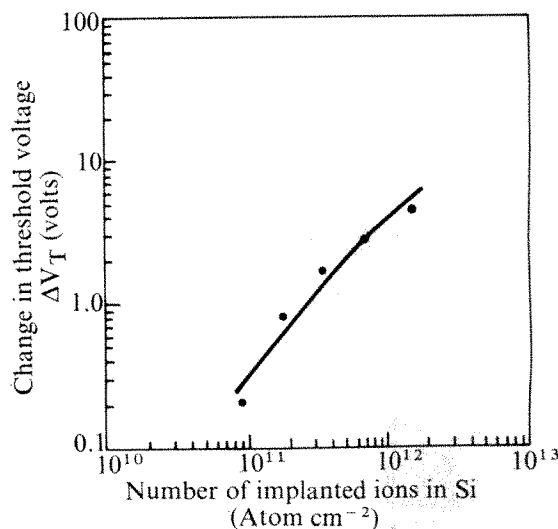
Fig. 4 An example of threshold control in an MOS transistor achieved by boron ion implantation (after Sigmon and Swanson[6]). 30 keV implant oxide thickness ≈ 550 Å boron, 500 °C anneal. ●, Experimental.

with a reproducible distribution of electrically active donors. Other improvements have come from a better control of the size of residual defects (Fig. 3) which remain after annealing, and which can, if large, project through the junction with undesirable local effects on conduction. By a careful choice of annealing schedule these defects can be reduced to a negligible 10 nm or so in size. Excellent wholly implanted bipolar transistors have thus been produced in many laboratories, and this has led to a considerable upsurge in interest in ion implantation, which is now seen as an important process for practically all new device fabrication.

Specialised diodes, such as the variable-capacitance devices used for tuning purposes in radio and TV, or avalanche photodiodes which are fast, sensitive optical detectors, or nuclear particle detector diodes are all made more controllably by ion implantation. Indeed, yield improvements of two orders of magnitude have been reported for variable-capacitance diodes. However, these structures account for only a small fraction of silicon device output. A more promising development is in high-power rectifiers, in which ion implantation provides a close control of doping within a very thin layer. This thin layer can store very few trapped charges and hence a fast recovery is achieved without the need for the usual gold doping. 100-A, 150-V ion-implanted rectifiers are already on the market, and it is forecast that the process will be useful for diodes operating at up to 3,000 A.

Compound semiconductor devices

At first it was widely believed that ion implantation would speedily advance the fabrication of compound semiconductor devices, because it is in these materials that thermal diffusion is least satisfactory. Unfortunately this has not come about, for several reasons. In compounds, ion bombardment can create a wider variety of defects in which, for example, atoms are located on the wrong lattice sites. Some of these defects, after clustering, are thermally very stable, and the compound becomes unstable at temperatures below that required to restore order.

The problem then becomes one of finding a suitable encapsulating material which will prevent loss of the more volatile constituent during annealing. In GaAs (the most widely-used compound semiconductor) it has not proved easy to find a practicable means of encapsulation, but progress is being made along several ingenious routes. A good deal of exploration has been carried out to find the most suitable dopants, and Si^{4+} ions have been shown to provide a high degree of donor activity[7].

More immediate success was achieved in the inverse direction, that is that of destroying electrical conductivity in selected areas of semiconductor by ion bombardment. Modest doses

(10^{14}–10^{15} cm^{-2}) of protons will render GaAs, GaP or InP semi-insulating, in a manner that is very useful for isolating one device from its neighbour. In GaP, for example, the bombardment also turns the material opaque, and this has been exploited in a light-emitting diode array in which the individual elements are isolated both optically and electrically (A. R. Peaker, unpublished paper delivered at the European Solidstate Device Research Conference, Nottingham, 1974). Proton-induced defects are not very stable, however, since they probably consist of very small disordered regions and they are rather easily annealed out. There is a need for a less empirical approach to this useful process.

Equipment for ion implantation

The design and manufacture of ion accelerators and target chambers for ion implantation, in research and production, has become a sizeable industry, with an estimated world market of over £100 million over the next five years.

Some of the more useful ion sources for implantation purposes were developed originally for the separation of isotopes, where a simple, rugged source capable of delivering intense currents of a variety of ion species is also required. For research purposes it is advantageous to be able to change from one ion beam to another without elaborate clean-up procedures, while for industrial applications the reliable and efficient production of a few important ion species is paramount.

There are two possible configurations for the acceleration system: in one case ions are mass-analysed after reaching their full energy while in the other magnetic analysis is carried out after acceleration through only 20–40 keV and the selected beam is further accelerated to the required energy. The latter has the advantages that the high-voltage supply need not be well stabilised, and only the specific ions required are accelerated so that X rays resulting from back-streaming secondary electrons are minimised. Either the magnetic analysis system or the target chamber must be at a high potential, however—a feature which can pose a few design problems. Both these arrangements have been adopted in systems based on the Lintott accelerator. This equipment delivers the highest currents of any commercial machine, and consideration has had to be given to the consequences of target heating since a 1 mA beam at 100 keV dissipates 100 W and this power is not readily conducted from semiconductor wafers in vacuum. The solution[8] has been to manipulate many wafers rapidly through the beam, so spreading the heat dissipation, while also achieving a high degree of uniformity in the dose (ions per unit area). In this way, a throughput of several hundred 5 cm-diameter silicon wafers can be achieved in a day, and the process becomes very economical. In most research facilities, however, the ion beam is swept electrostatically over the face of a single fixed specimen, but it is not easy to achieve such a high degree of uniformity in dose. For example, neutral atoms resulting from collisions with residual gas are undeflected and give a central spot in the distribution.

Accurate current measurement, in the presence of secondary electrons and secondary ions, is not a trivial matter and comparisons between results obtained on different implantation systems can be surprising. Work is in progress to develop an absolute calorimetric ion collector, and to provide means of calibrating accurately an implanted specimen against a laboratory standard, using an ion backscattering technique at MeV energies.

Ion implantation of metals

During the past year or two it has become clear that ion implantation has important applications outside the semiconductor field, and an investigation has begun of the effects of implantation upon the surface properties of metals and alloys. The corrosion behaviour, electrochemical properties, coefficient of friction, wear resistance and bonding ability of metals are controlled by their near-surface composition. Novel

Fig. 5 The effect of yttrium implantation in a niobium-containing steel alloy, compared with that of alloyed yttrium, on the oxidation of the metal in CO_2 at 700 °C (after Antill *et al.*[11]). ○, 20/25/Nb; □, 20/25/Nb 0.13% Y alloy; △, 20/25/Nb 0.41% Y alloy; ●▲, Y implanted 20/25/Nb.
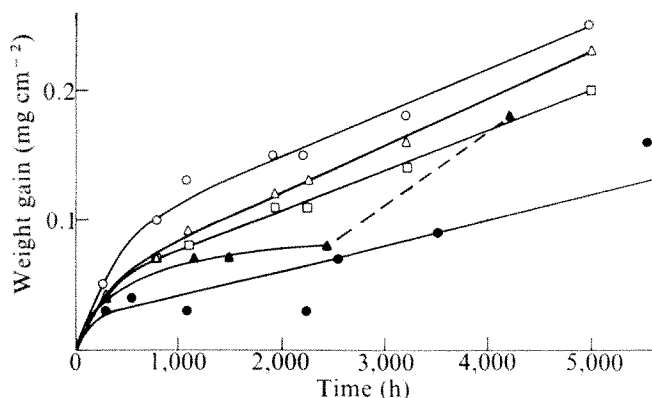
experiments on all these phenomena can be made by exploiting the ability to alter the surface composition in a versatile manner, and to monitor the migration of atoms initially confined to a shallow layer.

Thus at Harwell, we have studied the effects on thermal oxidation as a result of the implantation of a wide variety of ion species into titanium, zirconium and stainless steel[9]. The results in titanium and steel indicate a close correlation between the electronegativity of the impurity ions and the oxidation rate, but effects were reversed in the two metals. The behaviour of zirconium was quite different from that of titanium in spite of their proximity in the Periodic Table. There was no correlation with electrochemical properties but some indications of a trend with ionic size, which has been observed also in alloy studies. We inferred that these striking differences arise from the crystallographic properties of TiO_2 and ZrO_2. Crystallographic shear in TiO_2 is a powerful mechanism for eliminating oxygen vacancies, while in ZrO_2 the mechanical stress caused by volume changes upon oxidation is relieved by cracking and pore formation. The ionic size of impurities will influence this stress, and thus the degree of oxygen transport along fissures.

In these metals, bombardment by inert ions, or ions of the same species as the target produced little or no change in oxidation rate. But in the case of metals such as chromium or nickel, cation out-diffusion is the dominant process during oxidation and this seems to be inhibited by Cr^+ or Ni^+ bombardment, respectively.

This effect, together with the observation by Hartley[10] of a striking increase in wear resistance of ion-implanted steels, can tentatively be explained as a result of the lateral compressive stress generated by ion implantation. Such stresses can approach the yield stress of the material, and thus have a considerable influence on grain boundary phenomena and surface plasticity.

Besides these scientific studies, the practical possibility of

treating metallic components by ion implantation to improve their surface properties is arousing interest. Antill and others at Harwell have demonstrated[11] that implanted yttrium can be as effective as alloyed yttrium for inhibiting the high-temperature oxidation of an austenitic stainless steel in CO_2, without the drawbacks of a reduced bulk tensile strength and ductility. In this and other cases still under investigation the duration of the effects of ion implantation is remarkably long. This is due, in the case mentioned, to the fact that the yttrium remains near the metal–oxide interface. In other instances, and in wear modification, it could be that the initial phase of the corrosion or wear process is crucial in determining the subsequent pattern of behaviour.

In such practical applications the successful exploitation of ion implantation will depend upon economic factors, the geometry of the workpiece and the merits of alternative techniques. Novel configurations of ion accelerator and more versatile designs of ion sources will need to be developed, but the costs of the process seem likely to be acceptable for a variety of applications in both high and low cost systems.

1 Lindhard, J., Scharff, M., and Schiøtt, H. E., Kgl. Danske Vid. Selsk., Matt.-Fys. Medd., 33, no. 14 (1963).
2 Dearnaley, G., Freeman, J. H., Nelson, R. S., and Stephen, J., Ion Implantation, Ch. 2 (North-Holland, Amsterdam, 1973).
3 Allen, R. M., Proc. European Conf. on Ion Implantation, 127 (Peter Peregrinus, Stevenage, 1970).
4 Seidel, T. F., Meek, R. L., and Cullis, A. G., Proc. Int. Conf. on Lattice Defects in Semiconductors (Institute of Physics, London, in the press).
5 Sigmon, T. W., and Swanson, R., Sol. State Electronics, 16, 1217 (1973).
6 Amelio, G. F., Proc. Conf. on Charge-coupled Devices (University of Edinburgh Press, Edinburgh, 1974).
7 Woodcock, J. M., Shannon, J. M., and Clark, D. J., Sol. State Electronics, 18, 267 (1975).
8 Freeman, J. H., et al., Proc. Int. Conf. on Ion Implantation in Semiconductors and Other Materials, Osaka 1974 (in the press).
9 Dearnaley, G., Proc. Conf. on Applications of Ion Beams to Metals, 63 (Plenum Press, New York, 1974).
10 Hartley, N. E. W., Tribology, 65 (April 1975).
11 Antill, J. E., Bennett, M. J., Dearnaley, G., Fern, F., Goode, P. D., and Turner, J. F., Proc. Conf. on Ion Implantation in Semiconductors and Other Materials, 415 (Plenum, New York, 1974).

articles

Principles of protein–protein recognition

Cyrus Chothia & Joël Janin

Service de Biochimie Cellulaire, Institut Pasteur, 75015 Paris, France

The formation of the protein–protein interface by the insulin dimer, the trypsin-PTI complex and the αβ oxy-haemoglobin dimer removes 1,130–1,720 Å² of accessible surface from contact with water. The residues forming the interface are close packed: each occupies the same volume as it does in crystals of amino acids. These results indicate that hydrophobicity is the major factor stabilising protein–protein association, while complementarity plays a selective role in deciding which proteins may associate.

BIOLOGICAL molecules are able to recognise each other and form specific associations. These processes are fundamental

to biology and the nature of the interactions involved has frequently been discussed.

Recently X-ray diffraction analysis has provided detailed atomic information on several systems which involve protein–protein recognition and association. As a result of this we can now approach directly the problem of the nature of the interactions. We shall use here the known atomic structures of dimeric porcine insulin[1], of the bovine trypsin–pancreatic inhibitor complex[2,3] and of the αβ dimer of horse oxy-haemoglobin[4].

The association between protein subunits in these systems has been described in terms of Van der Waals' contacts, of electrostatic forces and of hydrogen bonds: that is, of point-to-point interactions. Here we analyse the contact regions between subunits in terms of surface area and of the volumes

occupied by residues, and show how these quantities, which can be interpreted from a thermodynamic point of view, account for the specificity and the stability of the associations.

Area and chemical nature of surfaces involved in protein–protein association

The concept of accessible surface area[5] describes the extent to which protein atoms can form contacts with the solvent. For a given atom it is defined as the area over which the centre of a water molecule can be placed while retaining Van der Waals' contact with that atom and not penetrating any other atom. Accessible surface areas are correlated with hydrophobic free energies[6].

Using available atomic coordinates and a computer program written by M. Levitt, we have calculated in the manner described previously[7], the total area accessible to water for each protein complex and for the isolated subunits. The surface area buried in the complex is then defined as the accessible surface area of one subunit plus that of the other subunit minus that of the complex (Table 1). This assumes that the free subunits have the same conformation as found in the complex. For the pancreatic trypsin inhibitor (PTI), the structure of which has been determined independently as the free molecule[8] and as the trypsin–PTI complex[2], we find that the small variations of conformation which do occur on binding have little effect on the estimation of the surface areas (Table 1).

Table 1 Changes in accessible surface area

Complex	Total accessible surface area (Å²)	Area buried in complex (Å²)	%	No. of residues involved*
Insulin dimer				
Monomer M1	3,400	530	16	12
Monomer M2	3,310	600	18	14
Total change		1,130		
Trypsin–PTI				
Trypsin	8,920	640	7	17
PTI	3,700	750†	20	12
Total change		1,390		
Horse oxyhaemoglobin				
α subunit	6,940	930	13	20
β subunit	7,130	790	11	19
Total change in α β		1,720		

*Number of residues contributing more than 10 Å² to the surface area buried in the complex. In all cases residues which contribute less than 10 Å² make up for 2–7% of the total buried area.

†Value obtained as described in text. A value of 815 Å² was calculated using atomic coordinates for free PTI rather than the PTI moiety of the complex.

For each of the three interactions studied here, the association of the protein subunits leads to a reduction of the surface area accessible to solvent by more than 1,000 Å². The chemical nature of the groups involved is analysed in Table 2, which details the relative contributions of polar and non-polar atoms to the accessible surface areas of the molecules and to the interfaces. Eight aromatic residues occur in the insulin dimer interface (Tyr B16, Phe B24, Phe B25 and Tyr B26, from each monomer) making that interface a good deal more hydrophobic than the rest of the protein surface. This is less true of the αβ interface in haemoglobin, while the trypsin–PTI interface has essentially the same chemical composition as other parts of the protein surface which remain accessible to the solvent. Thus, a number of polar or charged atoms become inaccessible to solvent when the complexes form: Table 3 shows that most, if not all, form hydrogen bonds.

Protein interfaces are close packed

Richards[9] has shown that the volume occupied by an atom inside a protein molecule could be defined by tracing an

Table 2 Chemical character of protein surface

Surface	Contribution to surface areas of		
	non-polar* (%)	polar† (%)	charged atoms (%)
Insulin			
Monomer M1	62	31	7
Monomer M2	59	33	8
Interface	74	22	4
Trypsin–PTI			
Trypsin	53	38	8
PTI	57	23	20
Interface	56	32	12
Oxyhaemoglobin			
α subunit	64	20	16
β subunit	58	23	19
Interface	68	16	16

*All C atoms.
†N, O and S atoms except in charged groups.

appropriate space-filling polyhedron (Voronoi polyhedron) separating that atom from adjacent atoms. This method was used to demonstrate that protein interiors are close packed[9] with individual amino acid residues occupying the same volume as they do in crystals of the amino acids themselves[7]. Using Richards' computer program in the manner described previously[9,7], we have calculated the volume occupied by residues buried in the interfaces: that is, by residues which are inaccessible to solvent in the complex but have a large accessible surface area in the isolated subunits. The results are summarised in Table 4, which also lists the volume occupied by residues in protein interiors and in crystals of amino acids. Table 4 shows clearly that residues occupy the same volume in protein interfaces, inside a protein or in the crystalline amino acid. The standard deviations are ~6% and a loose packing would cause a systematic tendency of the volumes towards higher values. The total volume of the residues cited in Table 4 for each complex, however, is in all cases within 1% of the corresponding volume calculated from crystalline amino acids. Thus the same rule applies to small organic molecules in the crystal state, and to amino acid residues in protein interfaces (or inside proteins): they close pack.

Free energy of protein association

The free energy of dissociation ΔG_D of a complex between two molecules is related to its dissociation constant K_D by:

$$\Delta G_D = -RT\ln K_D \qquad (1)$$

This equation implicitly refers to a standard state where all chemical species are 1 M, and attributes a free energy of zero to a complex with a dissociation constant of 1 M.

When two molecules associate, there is a loss of translational and rotational entropy which can easily be derived from

Table 3 Buried polar atoms and hydrogen bonds

Complex	No. of polar atoms buried in interface	Atoms buried forming H bonds	No. of new H bonds formed*
Insulin dimer	12	12	4†
Trypsin–PTI	29	25‡	13
Haemoglobin α β dimer	31	27§	10
Total	72	64 (89%)	

*Requirements for H bonds include proper orientation of groups and a maximum distance of 3.7 Å (in fact all but four are within 3.3 Å).
†These bonds have been described in ref. 1.
‡Exceptions are: Sγ Cys 42 of trypsin, Sγ Cys 14, O Ala 16 and O Arg 17 on PTI.
§Possible exceptions are: Nε His α103, Nδ and Nε His α122, and Sδ Met β55. More recent data (R. Ladner, personal communication) suggest that the first three are in fact hydrogen bonded.

statistical thermodynamics (refs 10 and 11 and our unpublished work) at least in the case of perfect gases. While this might appear quite irrelevant to macromolecules in aqueous solutions, Page and Jencks[11] have shown that the calculation gives reasonable estimates of the entropy lost by small organic molecules reacting in solution. Applying it now to the complexes studied here, we find that the entropy corresponding to three translational and three rotational degrees of freedom lost when two molecules associate amounts to 70–100 calorie $deg^{-1}mol^{-1}$, equivalent to a free energy, ΔG^s_D, of 20 to 30 kcalorie mol^{-1} at 27 °C (in the standard state). To form a complex with a

Table 4 Volume of residues buried in subunit interfaces

Residue	No.	Volume			
		V_S*	σ of V_S	V_P†	V_H‡
Gly	7	68.5	6.6	66.4	66.5
Phe	6	196.3	7.8	203.4	—
Ala	5	89.4	6.9	91.5	96.6
Val	5	141.1	6.7	141.7	143.4
Pro	5	128.9	11.7	129.3	124.4
Leu	2	161.3	9.4	167.9	—
Ile	1	175.2	—	168.8	169.7
Ser	4	99.7	7.0	99.1	102.2
Tyr	3	205.1	9.6	203.6	201.7
Cys	2	108.9	1.7	105.6	108.7
Trp	1	241.6	—	237.6	—
His	4	167.6	13.5	167.3	166.3
Gln	4	165.5	7.0	161.1	148.0
Asn	1	147.4	—	135.2	—
Arg	3	202.1	3.2	—	—
Lys	2	172.9	1.3	171.3	—
Asp	1	127.2	3.7	124.5	122.0

*Average volume of residue in protein interfaces.
All residues contributing more than 10 $Å^2$ to the buried surface area and having less than 20% of their surface accessible in the complex are included.
†The average volume of the residue when buried in the protein interior[7].
‡The volume of the residue when in crystals of the amino acid[7].

dissociation constant K_D, one must therefore provide a free energy at least equal to:

$$\Delta G^t_D = \Delta G^s_D + \Delta G_D \qquad (2)$$

Table 5 shows that weak but observable protein–protein interactions such as found in the insulin dimer require at least 25–35 kcalorie mol^{-1} of free energy, while the tight binding of PTI to trypsin or of the α and β subunits in haemoglobin needs 30–50 kcalorie mol^{-1}.

Origin of free energy of association

Can the structural analysis of the protein interfaces described above account for these large energies? What relative contribution does each type of interaction make to the association? Pauling[15] has argued that energy of association by proteins is due to the complementarity in structure and the resultant cooperation of weak forces (that is, H bonds and Van der Waals' interactions) over a sufficiently large area to produce a strong bond. An alternative model, due to Kauzmann[16], stresses the importance of hydrophobic energies, the association areas of proteins being viewed as surface patches of non-polar side chains brought together rather like hydrocarbon chains in lipid micelles.

Hydrogen bonds and Van der Waals' interactions

Thermodynamic evidence is against hydrogen bonds making a major contribution to the free energy of protein–protein interaction[17,18]. Although the enthalpy of breaking a typical amide H bond may be of the order of 4–5 kcalorie mol^{-1}

(ref. 19), the difference between a protein–protein H bond and the corresponding bond made with water in the dissociated complex can only be a fraction of this. It is unlikely that the four H bonds of the insulin dimer or even the 13 H bonds of the trypsin–PTI complex contribute more than 4–15 kcalorie mol^{-1} to the dissociation free energies.

Van der Waals' interactions are both much less energetic[20] and much more numerous than H bonds as they involve all pairs of neighbouring atoms. But as atoms making Van der Waals' interactions in the complex make similar interactions with water in the dissociated subunits, the overall contribution is small. Still, like hydrogen bonds, Van der Waals' interactions place severe restrictions on the structure of protein surfaces: to achieve the same interactions with each other as they do with water, the two surfaces must be complementary, and this is expressed in the close packing of residues, which we have described.

Hydrophobic contribution

We have ignored so far the effect of solvent entropy on the thermodynamic balance of the association: that is, the hydrophobic contribution. For this, we shall use an empirical correlation found between the accessible surface area of a solute and its free energy of transfer to a water solution. Such a correlation has been established for hydrocarbons and amphiphilic molecules[21,22], and also for amino acids[6]: for the latter, 1 $Å^2$ of surface area corresponds to 25 calorie mol^{-1} of hydrophobic free energy[7].

A free energy term favouring association is thus derived from the decrease in surface area accessible to water occurring when proteins associate. Using the values of Table 1, we calculate the hydrophobic contribution to the formation of the insulin dimer, of the PTI–trypsin complex and of the αβ dimer of haemoglobin. Table 5 shows that this contribution is very large in each case, and of the same order as the value of ΔG^t_D estimated above: in other words, the entropy gained by water due to the smaller accessible protein surface area is sufficient to compensate the entropy loss by protein molecules forming a complex.

Stability and complementarity

These results show that hydrophobicity is the major factor which stabilises protein–protein associations. Our estimates of the free energy required to make a stable complex indicate that each subunit needs to bury about 600 $Å^2$, corresponding to a hydrophobic contribution of 30 kcalorie mol^{-1} (for two subunits). In the case of the trypsin inhibitors this surface

Table 5 Free energy of protein–protein association

Complex	Insulin dimer	Trypsin–PTI	Haemoglobin αβ dimer
Dissociation constant K_D (molar)†	10^{-5}	10^{-13}	$\ll 10^{-8}$
Free energy of dissociation ΔG_D (kcalorie mol^{-1})†	7	18	>11
Translational/rotational free energy‡ ΔG^s_D (kcalorie mol^{-1})	23	27	27
Free energy required for association ΔG^t_D (kcalorie mol^{-1})§	30	45	>38
Surface area buried in the complex ($Å^2$)	1,130	1,390	1,720
Hydrophobic free energy (kcalorie mol^{-1})¶	28	35	43

*Data at neutral pH near 2 °C from refs [12–14].
†$\Delta G_D = -kT \ln K_D$.
‡Calculated from the translational/rotational partition functions.
§$\Delta G^t_D = \Delta G^s_D + \Delta G_D$.
¶25 calorie mol^{-1} per $Å^2$ of buried surface area.

area is derived from only a small number of amino acid residues (ref. 3 and our unpublished work). As the hydrophobic contribution is entirely unspecific, it would lead to all kinds of incorrect interactions in a cell. The proper formation of hydrogen bonds and of Van der Waals' contacts, however, requires complementarity of the surfaces involved: they must be able to close-pack together, with the polar atoms properly positioned to make hydrogen bonds. Thus while Van der Waals' and polar interactions contribute little to the stability of the complex, they decide which proteins may recognise each other. Incorrect associations are forbidden by large unfavourable enthalpies due to poor packing and loss of hydrogen bonds made to water. These principles are likely to be also true of the interactions of proteins with small ligands, nucleic acids, and membranes.

We thank Frederic M. Richards and Michael Levitt for discussion and computer programs, and Drs G. Dodson, D. Hodgkin, R. Huber, B. Heidner and M. F. Perutz for atomic coordinates.

1 Blundell, T., Dodson, G., Hodgkin, D., and Mercola, D., *Adv. Protein Chem.*, 26, 279–402 (1972).
2 Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., and Steigemann, W., *J. molec. Biol.*, 89, 73–101 (1974).
3 Rühlmann, A., Kukla, D., Schwager, P., Bartels, K., and Huber, R., *J. molec. Biol.*, 77, 417–436 (1973).
4 Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G., *Nature*, 219, 131 (1968).
5 Lee, B., and Richards, F. M., *J. molec. Biol.*, 55, 379–400 (1971).
6 Chothia, C. H., *Nature*, 248, 338–339 (1974).
7 Chothia, C. H., *Nature*, 254, 304–308 (1975).
8 Deisenhofer, M., and Steigemann, W., *Second Int. Res. Conf. Proteinase Inhibitors*, (Springer, Heidelberg, 1974).
9 Richards, F. M., *J. molec. Biol.*, 82, 1–14 (1974).
10 Doty, P., and Myers, G. E., *Discussion Faraday Soc.*, 13, 51 (1953).
11 Page, M. I., and Jencks, W. P., *Proc. natn. Acad. Sci. U.S.A.*, 68, 1678–1683 (1971).
12 Pekar, A. H., and Frank, B. H., *Biochemistry*, 11, 4013–4016 (1972).
13 Vincent, J. P., and Lazdunski, M., *Biochemistry*, 11, 2967–2977 (1972).
14 Kellett, G. L., and Schachman, H. K., *J. molec. Biol.*, 59, 387–399 (1971).
15 Pauling, L., and Pressman, D., *J. Am. chem. Soc.*, 67, 1003–1012 (1945).
16 Kauzmann, W., *Adv. Protein Chem.*, 14, 1–63 (1959).
17 Schellman, J. A., *C.r. Trav. Lab. Carlsberg, Serv. Chim.*, 29, 223–229 (1955).
18 Klotz, I. M., and Franzen, J. S., *J. Am. chem. Soc.*, 84, 3461–3466 (1962).
19 Kresheck, G. C., and Klotz, I. M., *Biochemistry*, 8, 8–12 (1969).
20 Maitland, G. C., and Smith, B. B., *Chem. Soc. Rev.*, 2, 181–210 (1973).
21 Hermann, R. B., *J. phys. Chem., Ithaca*, 76, 2754 (1972).
22 Reynolds, J. A., Gilbert, D. B., and Tanford, C., *Proc. natn. Acad. Sci. U.S.A.*, 71, 2925–2927 (1974).

Chloroplast DNA from higher plants replicates by both the Cairns and the rolling circle mechanism

Richard D. Kolodner* & K. K. Tewari

Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92664

The chloroplast DNA (ctDNA) from pea and corn plants contains both Cairns type and rolling circle replicative intermediates. Denaturation mapping studies with pea ctDNA molecules have shown that the rolling circles initiate replication at or near the site where the Cairns replicative intermediates terminate replication. These results suggest that the rolling circles are initiated by a Cairns round of replication. A model for the replication of the chloroplast DNA is based on these results.

THE chloroplast DNAs from higher plants and the alga *Euglena* have been isolated as covalently closed circular molecules with molecular weights from 85×10^6 to 96×10^6 (refs 1–3). The replication of corn and pea ctDNA[4,5] has been shown to initiate with the formation of two displacement loops (Fig. 1b) similar to the displacement loops that initiate animal mitochondrial DNA (mtDNA) replication[5,6]. The two displacing strands are hydrogen bonded to the opposite parental DNA strands, they expand toward each other (Fig. 1c), and form a structure that looks like a Cairns replicative intermediate when the two displacing strands elongate past each other (Fig. 1d). We present here evidence that pea and corn ctDNA replicate by both the Cairns replication mechanism[8] and by the rolling circle mechanism[9]. Electron microscopic examination of pea and corn ctDNA have shown the presence of Cairns type and rolling circle type structures in both ctDNAs. Denaturation mapping studies on pea ctDNA have shown that the Cairns replicative intermediates elongate bidirectionally. The de-

naturation mapping experiment revealed that all the rolling circles initiated replication at the same site but could elongate in either direction from that site. The initiation site for the rolling circle round of replication was found to lie at or near the termination site of the Cairns round of replication, suggesting that the rolling circle replicative intermediates are initiated by the Cairns round of replication.

Structure of intermediates

To obtain replicative intermediates, the pea ctDNA was fractionated by centrifugation in ethidium bromide-caesium chloride density gradients[3,7]. The DNA was collected into three fractions; the lower band of closed circular DNA containing 20–25% of the total pea ctDNA, the middle band
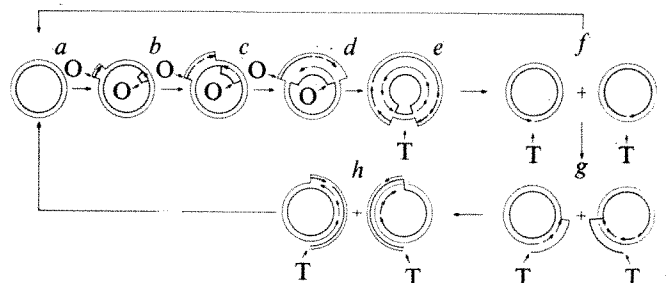
Fig. 1 A model for the replication of ctDNA. *a*, Closed circular parental molecule; *b*, D-loop containing molecule; *c*, expanded D-loop containing molecule; *d* and *e*, Cairns type of replicative intermediate; *f*, nicked progeny molecules; *g* and *h*, rolling circles. The thin and thick lines mark the opposite strands of a molecule. The lines with the arrows are the daughter strands. O indicates the positions of the two origins of D-loop synthesis which are 5.2% of pea ctDNA apart. T indicates the terminus of the Cairns round of replication which is 180° opposed to the origins of D-loop synthesis.

containing 10% of the pea ctDNA, and the upper band of nicked circular and linear DNA containing 65–70% of the pea ctDNA (60% of the upper band DNA was circular). The upper band DNA was determined to be ctDNA by its buoyant density in CsCl and its renaturation rate[3]. These fractions were examined in the electron microscope using the formamide
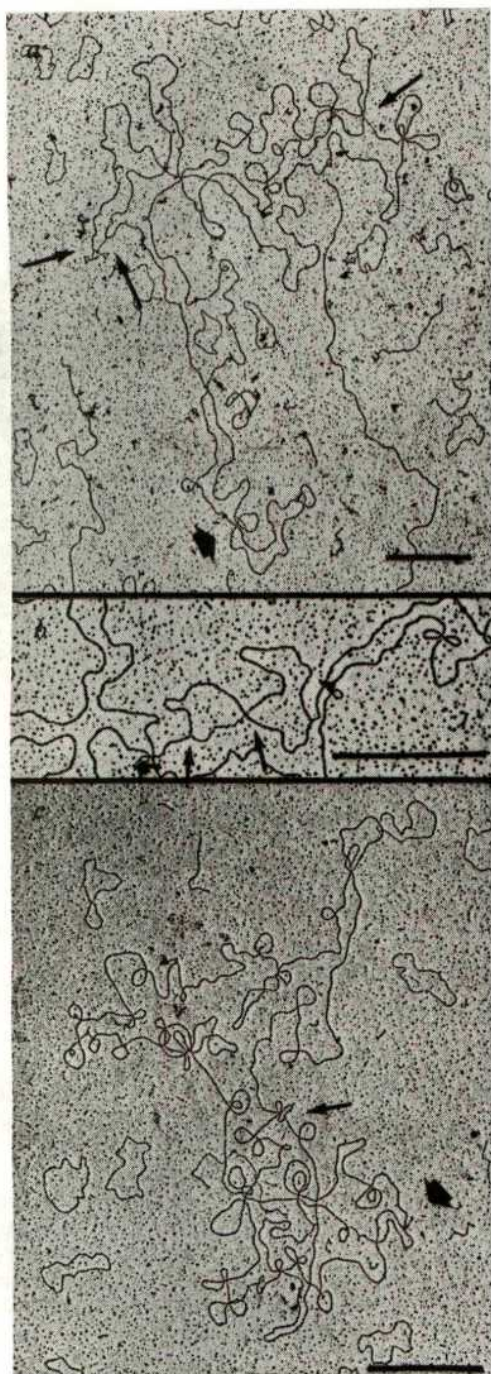
Fig. 2 Cairns replicative intermediates. *a*, A molecule that is 37% replicated and contains a single strand region (two small arrows) at one growing fork and a denatured region (large arrow) in the unreplicated portion of the molecule. *b*, A portion of a molecule that is 5.2% replicated and contains a single strand region (two small arrows) at one growing fork. *c*, A Cairns replicative forked molecule showing branch migration at one of the replicative forks (large arrow). The molecules were mounted for electron microscopy by the formamide technique[10,18]; the spreading solution contained 50% formamide, 0.1 M Tris, 0.01 M EDTA, *p*H 8.5, the hypophase contained 20% formamide, 0.01 M Tris, 0.001 M EDTA, *p*H 8.5, and the spreading was performed at 23 °C. Single-stranded and double-stranded ΦX DNA molecules were used as internal standards in all experiments. The bars indicate 1 μm.
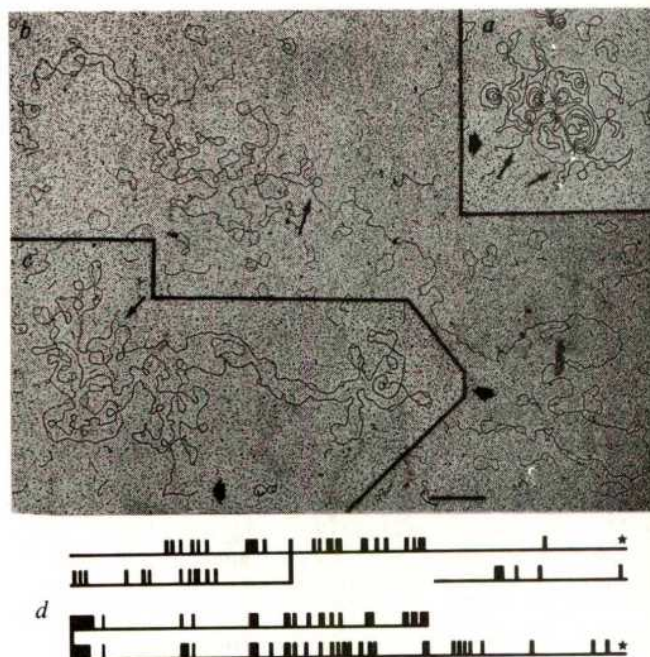
Fig. 3 Rolling circle molecules. *a*, A molecule with a tail that is 19% of the length of the attached circle. There is a single-strand region at the growing fork that is 3 500 bases long (indicated by arrows). This molecule was mounted for electron microscopy using the conditions described in Fig. 2 *b* and *c*, Partially denatured molecules with tails that illustrate the two different denatured patterns at the tip of the tails. These molecules were mounted for electron microscopy under partially denaturing conditions using the formamide technique as previously described[18]. The spreading solution contained 77% formamide, 0.1 M Tris, 0.01 M EDTA, *p*H 8.5, the hypophase contained 47% formamide, 0.01 M Tris, 0.001 M EDTA, *p*H 8.5, and spreadings were performed at 23 °C. Single-stranded and double-stranded ΦX DNA molecules were used as internal standards in all experiments. *a*, Graphical representation of molecules in *b* and *c*. The boxes indicate the denatured regions, the asterisk indicates the circular part of the molecule that has been linearised. The unmarked free end is the tip of each tail. The bars indicate 1 μm.

technique to demonstrate single-stranded DNA[10]. Cairns type replicative forked structures were found in each fraction from two different DNA preparations (Fig. 2). Forty-five Cairns structures were measured and the extent of their replication ranged from 5.6 to 87%. Three per cent of the circular molecules in the lower band were Cairns structures and the extent of their replication ranged from 5.2 to 8.2%. The Cairns replicative intermediates made up an average of 5.7% of the circular pea ctDNA molecules in the middle band and the extent of their replication ranged from 7 to 50%. The Cairns structures consisted of 2.9% of the circular pea ctDNA molecules in the upper band and the extent of their replication ranged from 38% to 87%. In this study 3,118 circular pea ctDNA molecules were scored and it was calculated that 3.3% of the total circular pea ctDNA molecules consisted of Cairns replicative intermediates. The finding of Cairns replicative intermediates in the lower and middle bands of the caesium chloride–ethidium bromide density gradients, and the correlation between higher banding position and larger amounts of replication suggests that replication takes place on a covalently closed circular template, and is accompanied by nicking and closing cycles. Similar observations have been made in studies of the replication of mouse L cell mtDNA[11] and SV40 DNA[12].

The replicative intermediates of corn ctDNA were also studied. The total corn ctDNA was collected as a single fraction and 309 circular molecules were scored. Cairns replicative structures made up 4.5% of the total circular corn ctDNA molecules and the extent of their replication ranged from 9.4% to 70%. Cairns structures have also been found in *Euglena* ctDNA[13].

In the above experiments, monomer-length circular molecules with an attached double-stranded tail were also observed (Fig. 3a). In the pea ctDNA preparation, circular molecules with tails accounted for 4.9% of the circular molecules in the upper band, and 2.8% of the circular molecules in the middle band. No circular molecules with tails were observed in the lower band of pea ctDNA (<0.05%). The circular molecules with double-stranded tails made up 3.1% of the total circular pea ctDNA. The lengths of tails in the pea ctDNA ranged from 1.5 to 124% of the length of the attached monomer-length circular molecule. Circular molecules with double-stranded tails were also found in the preparation of total corn ctDNA that was examined. In corn ctDNA, 11% of the total circular molecules had tails attached to them. The tails in the corn ctDNA ranged in length from 2% to 140% of the attached monomer length circular molecule. Fifteen per cent of all rolling circle forms had tails longer than the unit length of ctDNA. The finding of tails that were longer than the attached monomer length circular molecule eliminates the possibility that the tails arose by breakage of a Cairns forked structure at a replicative fork. This suggests that the circular molecules with tails are rolling circle replicative intermediates[9].

Single-stranded regions in intermediates

A careful examination of the rolling circles and the Cairns forked structures in both pea and corn ctDNA showed that 77 to 81% of the growing forks in these molecules contained single-stranded regions (Figs 2a and b, and 3a). The lengths of these single-stranded regions were variable and ranged in length from 250 bases to 7,600 bases. The structure and frequency of these growing forks is presented in Fig. 4. In the Cairns structures, single-stranded regions could be observed at both of the replicative forks in a given molecule, which suggests that the Cairns structures grow bidirectionally. In unambiguously interpretable Cairns structures that contained single-stranded regions at both replicative forks, the single-stranded regions were located at positions that were *trans* with respect to each other (Fig. 4). In both the rolling circles and the Cairns structures, the single-stranded regions were located on only one daughter strand at each replicative fork. The single-stranded regions at the rolling circle growing forks were always found on the tail. These results indicate that at least one daughter strand at each growing fork is being synthesised discontinuously. The above observations do not eliminate the possibility that both daughter strands at each fork are being extended discontinuously, as in the case of T7 DNA[14] or *E. coli* DNA[15], since the failure to see gaps on both daughter strands of a growing fork would simply be a consequence of the 5' to 3' direction requirement of DNA synthesis[16].

Short single-stranded tails were observed (Fig. 2c) at some of

Fig. 4 Diagrammatic representation of the growing forks. *a* and *b*, Cairns replicative forks; *c*, rolling circle replicative forks. The stars indicate the parental part of the Cairns replicative intermediates.

		%	%	%	%	%	N
a	Pea	63	7	11	19	0	90
	Corn	50	15	15	20	0	28
		cis		*trans*			

		%	%	N
b	Pea	0	100	15
	Corn	0	100	6

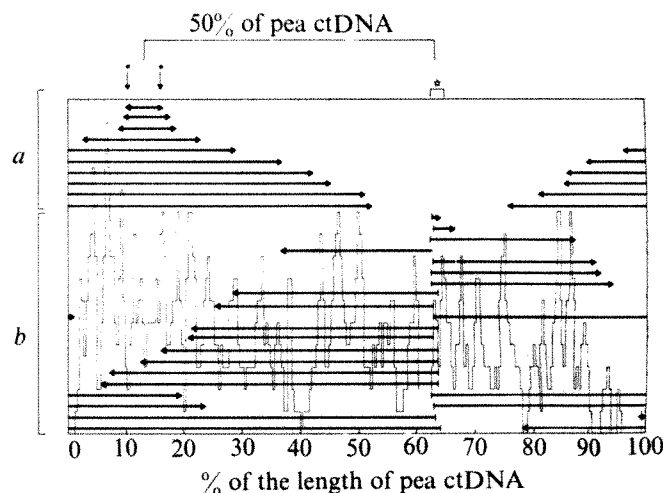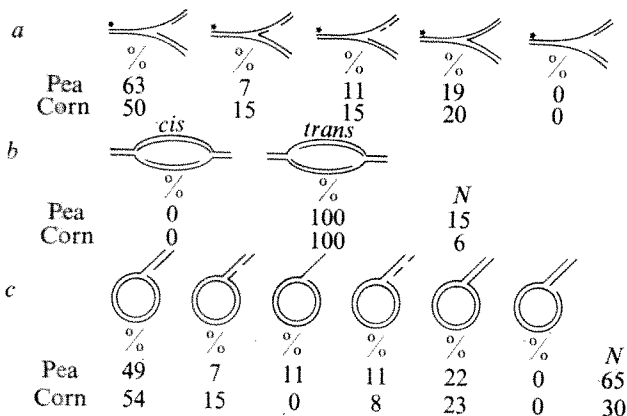|		%	%	%	%	%	%	N
c	Pea	49	7	11	11	22	0	65
	Corn	54	15	0	8	23	0	30

Fig. 5 Denaturation mapping of the replicative intermediates. DNA fractions containing replicative intermediates were mounted for electron microscopy under conditions of partial denaturation as described in the legend of Fig. 3. Under these conditions an average of 22% of each DNA molecule is denatured[18]. Three denaturation maps were constructed as described before[18] from the clean circular ctDNA molecules, from the circular part of ctDNA molecules containing double-stranded tails, and from the Cairns type of forked molecules. All three denaturation maps were very similar showing that all the molecules examined had an identical sequence. An example of such a denaturation map is represented by the histogram in this figure. The orientation of the replicated portion of 10 Cairns replicative intermediates in this denaturation map is represented by the horizontal lines (*a*). The arrows at the ends of the lines indicate the positions of the replicative forks. The tails of the rolling circle molecules were oriented in the denaturation map by comparing the denaturation pattern of the tail with both the denaturation pattern of the attached monomer length circular molecule and the denaturation map essentially as described by Schnös and Inman[19]. The orientation of the tails from 20 rolling circles are represented by horizontal lines (*b*). The single arrow on each line indicates the position in the denaturation map where the tail was attached to the circular molecule and represents the position of the replicative fork. The other end of the horizontal line (*b*) marks the position in the denaturation map where the end of each rolling circle tail maps. The small stars indicate the two origins of D-loop synthesis and the larger star indicates the origin of the rolling circle synthesis.

the replicative forks in pea and corn ctDNA replicative intermediates. These whiskers have been suggested to arise from branch migration at the replicative fork[17] and have been observed in the replicative intermediates from various systems[15]. Whiskers were observed at 17 and 15% of the rolling circle growing forks in pea and corn ctDNA, respectively. The Cairns structures in pea and corn ctDNA had whiskers at 13 and 15% of the growing forks, respectively. In addition to the whiskers, 12% of the pea ctDNA Cairns replicative intermediates found in the middle and lower bands had denatured regions in the unreplicated portion of the molecule (Fig. 2a). The relationship between branch migration and denatured regions in covalently closed replicative intermediates has been discussed elsewhere[7].

Denaturation mapping experiments

To study the pattern of the elongation of the Cairns forked structures and rolling circles, pea ctDNA was mounted for electron microscopy under conditions of partial denaturation[18]. Ten partially denatured Cairns replicative intermediates were ordered with respect to their position in the denaturation map of pea ctDNA molecules that were an average of 22% denatured[18]. The positions of the replicated portions of these molecules with respect to the denaturation map are illustrated in Fig. 5a. These partially replicated molecules have a pattern that would be expected if they all had initiated replication at the same place and that both replicative forks were being extended at the same rate. The initiation site of these Cairns replicative

intermediates lies at the same point in the denaturation map where displacement loops (Fig. 1b) were shown to map[7]. The termination site for the Cairns round of replication appears to lie at a site 180° around the pea ctDNA molecule from the initiation site (Fig. 5a).

The pea ctDNA rolling circles have also been studied by denaturation mapping. In the partially denatured pea ctDNA preparations, rolling circles with two distinct patterns of denaturation on the tip of the tail were observed. An example of each type is presented in Fig. 3. The two types of rolling circles appeared in equal proportion and no other types of denatured tails were seen. The two partially denatured rolling circles of Fig. 3b and c are represented as a line graph in Fig. 3d. This representation shows that the tip of the tail of these two molecules is mapped at the same site and that each tail extends in the opposite direction from this site. The positions of 20 partially denatured tails with respect to the pea ctDNA denaturation map are illustrated in Fig. 5. All the tips of the tails are mapped at the same site and the tails extend in either direction on the map from this site. In the rolling circle model of DNA replication the tip of the tail marks the site where the initiation of replication takes place[9]. The initiation of rolling circle replication at a unique site in the pea ctDNA is similar to the results found for E. coli phage P2 replication except that the P2 rolling circles elongate in only one direction[19]. It should be noted that the pea ctDNA rolling circles all initiate replication at a site that is at or near the site where the Cairns round of pea ctDNA replication was found to terminate, which suggests a connection between the two processes.

Model for replication of chloroplast DNA

The results presented here suggest a model for the replication of pea and corn ctDNA. This model is illustrated in Fig. 1. The ctDNA replication is initiated by the formation of two displacement loops whose displacing strands are complementary to the opposite parental strands of ctDNA (Fig. 1b). The two displacement loops expand towards each other and initiate the formation of a Cairns replicative forked structure (Fig. 1c and d). This initial stage of ctDNA replication has been discussed in detail[7]. The small Cairns forked structures expand bidirectionally (Fig. 1e) until termination takes place at a site that is 180° around the circular ctDNA molecule from the initiation site. Separation of the daughter molecules takes place yielding two circular molecules that each have a single-strand break or small gap at the same site located in opposite daughter strands (Fig. 1f). This part of the replication process is not well understood in any system, but it probably yields open circular progeny which are then sealed into closed circles[20]. In ctDNA the nicked circles could be sealed to closed circles or the 3'-OH of each nicked progeny molecule could be extended by a DNA polymerase molecule. This would displace a single-stranded tail (Fig. 1g) from the molecule and this tail could be filled in by discontinuous duplex synthesis to yield a molecule with a double-stranded tail (Fig. 1h). The tails might then be

converted to circular molecules by an intrastrand recombination event[21]. If both progeny from a Cairns round of replication initiated rolling circle synthesis, two types of rolling circles would be formed. In each case, the very tip of the tail would be mapped at the same site but the sequence of the two types of tails would extend in opposite directions from this site. This is the result that was observed in the denaturation mapping experiments with pea ctDNA rolling circles. Therefore, we feel that a Cairns round of replication is used to initiate a rolling circle round of replication in the ctDNA. A low frequency of Cairns structures was observed among the rolling circles that were shown to amplify the rDNA from Xenopus laevis[21] and both Cairns structures and rolling circles have been observed during E. coli phage λ DNA replication[22,23], suggesting a similar model of replication for these two DNAs.

It is interesting that the ctDNAs from pea and corn, two evolutionarily divergent plants, have similar modes of replication. Why should the higher plant ctDNAs require two modes of DNA replication, one of which is an amplification mechanism[21], and the other which is a duplication mechanism? Possibly it is related to a developmental aspect of higher plant chloroplast biogenesis that requires the rapid synthesis of a number of copies of ctDNA. The rolling circle mechanism might be used for this rapid synthesis of ctDNA while the Cairns mechanism might be used for normal duplication of ctDNA.

1 Kolodner, R., and Tewari, K. K., J. biol. Chem., 247, 6355–6364 (1972).
2 Manning, J. E., Wolstenholme, D. R., Ryan, R. B., Hunter, J. A. and Richards, O. C. Proc. natn. Acad. Sci. U.S.A., 68, 1169–1173 (1971).
3 Kolodner, R., and Tewari, K. K., Biochim. biophys. Acta (in the press).
4 Kolodner, R., and Tewari, K. K., J. Cell Biol., 59, 174a (1973).
5 Arnberg, A., Van Bruggen, E. F. J., Ter Schegget, J., and Borst, P., Biochim. biophys. Acta, 246, 353–357 (1971).
6 Kasamatsu, H., Robberson, D. L., and Vinograd, J., Proc. natn. Acad. Sci. U.S.A., 68, 2252–2257 (1971).
7 Kolodner, R., and Tewari, K. K., J. biol. Chem. (in the press).
8 Cairns, J., Cold Spring Harbor Symp. quant. Biol., 28, 43–46 (1963).
9 Gilbert, W., and Dressler. D., Cold Spring Harbor Symp. quant. Biol., 33, 473–484 (1968).
10 Davis, R. W., Simon, M., and Davidson, N., in Methods in Enzymology (edit. by Grossman, L., and Moldave, K.), 21, part D, 413–428 (Academic, New York, 1971).
11 Robberson, D. L., and Clayton, D. A., Proc. natn. Acad. Sci. U.S.A., 69, 3810–3814 (1972).
12 Sebring, E. D., Kelly, T. J., Thoren, M. M., and Salzman, N. P., J. Virol., 8, 478–490 (1971).
13 Richards, P. C., and Manning, J. E., in Euglena gracilis, Colloque International Du CNRS (in the press).
14 Masamune, Y., Frenkel, G. D., and Richardson, C. C., J. biol. Chem., 246, 6874–6879 (1971).
15 Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., Kainuma, R., Sugino, A., and Iwatsuki, N., Cold Spring Harbor Symp. quant. Biol., 33, 129–143 (1968).
16 Richardson, C. C., in A. Rev. Biochem., 38, 795–840 (1969).
17 Younghusband, H. B., and Inman, R. B., A. Rev. Biochem., 43, 605–618 (1974).
18 Kolodner, R., and Tewari, K. K., J. biol. Chem., 250, 4888–4897 (1975).
19 Schnös, M., and Inman, R. B., J. molec. Biol., 55, 31–38 (1971).
20 Kasamatsu, H., and Vinograd, J., A. Rev. Biochem., 43, 695–719 (1974).
21 Hourcade, D., Dressler, D., and Wolfson, J., Proc. natn. Acad. Sci. U.S.A., 70, 2,926–2,930 (1973).
22 Skalka, A., Poonian, M., and Bartel, P., J. molec. Biol., 64, 541–550 (1972).
23 Schnös, M., and Inman, R. B., J. molec. Biol., 51, 61–73 (1970).

letters to nature

Soft X rays from Sirius

ON April 3, 4 and 5, 1975 the star Sirius (α CMa) was observed with the soft X-ray detector aboard the Astronomical Netherlands Satellite (ANS). The instrument consists of a parabolic collecting mirror which has at its focus a proportional counter with a 3.6-μm polypropylene window. It responds to X rays in the photon energy range 0.284–~0.2 keV. The projected area is 144 cm[2] which, allowing for reflectivity, counter efficiency

and so on, leads to a sensitive area of about 25 cm[2] at the carbon absorption edge (0.284 keV). The field of view is 34' FWHM circular. Further instrumental details are given in refs 1–3.

So that background could be subtracted, the observations were carried out in the offset pointing mode with the instrument alternately pointed at the source for 64 s and 40' away from the source for 64 s, the transition time being 16 s. Any ultraviolet contamination by light from the star can be determined by interposing into the light path a 0.5-mm MgF₂ filter which
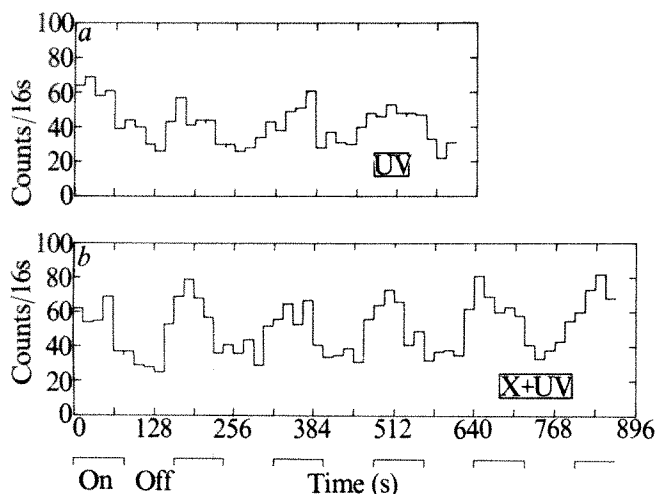
Fig. 1 Counting rates of Sirius measured on April 4, 1975 with ANS soft X-ray (0.25 keV) detector in offset pointing mode: *a*, 0920 UT; *b*, 0930 UT. UV (X+UV), signal measured with (without) MgF₂ filter; on (off), on (off) source.

blocks out the X rays but which transmits ultraviolet radiation above about 1,100 Å. The photons responsible for the ultraviolet contamination come from the wavelength region between about 1,600 and 2,500 Å (because of the transmission properties of the foil plus coating, ultraviolet radiation below 1,600 Å can be neglected). By measuring twice, with and without filter, X radiation (X) can be distinguished from ultraviolet radiation (UV). In that way the ultraviolet response was determined by observations on a number of bright ultraviolet stars (R.M. *et al.*, unpublished). Figure 1 shows typical measuring signals on Sirius, with and without the filter. In Fig. 2 the source signal, corrected for background and averaged over the total observation time of about 1,300 s, has been plotted against the relative ultraviolet flux. The total counting rate from Sirius, without the filter, that is, signal X+UV, is more than 6σ above the observed ultraviolet counting rate. The excess counting rate has been interpreted in terms of X-ray flux from that star system (R.M. *et al.*, unpublished). Figure 2 also shows that

Fig. 2 Counting rates of various ultraviolet stars as a function of the relative ultraviolet flux at 2,000 Å after subtraction of background. Vertical error bars are 2σ in length and represent the combined uncertainty in the source and background data. The excess signals {(X+UV)−UV} of Sirius (α CMa) and Capella (α Aur) are 6σ and 5σ, respectively. ×, Signal without ultraviolet filter; ⊙, signal with ultraviolet filter; arrow, ultraviolet response.
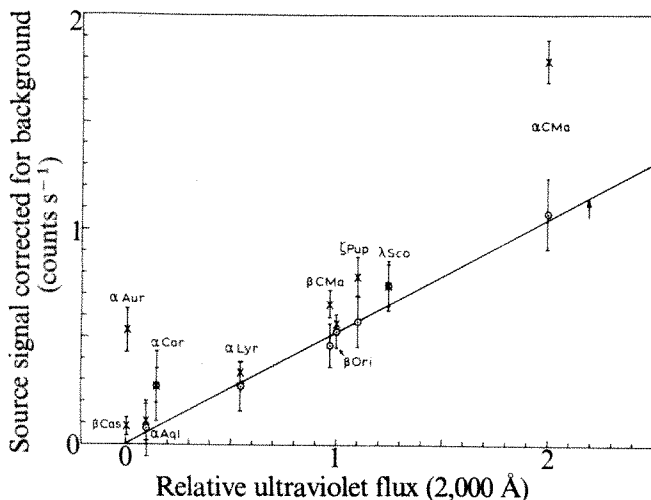
Capella (α Aur), detected earlier as an X-ray star[4], gives a signal 5σ above background.

The counting rates from the other stars depicted in Fig. 2 can be wholly attributed to ultraviolet radiation. The observed counting rate of 0.58 ± 0.10 counts s⁻¹ and the instrumental sensitivity of 1.9 counts (photon cm⁻² keV⁻¹)⁻¹ give for Sirius an X-ray luminosity in the energy band 0.284–0.2 keV: $L_X = (9.1 \pm 1.6) \times 10^{27}$ erg s⁻¹. Sirius is a visual binary with at least two components: Sirius A (A1 V) and Sirius B (white dwarf). The rotation period is 50 yr, the present relative distance, *a*, of the two components is estimated to be about 30 AU.

The observations could be explained by invoking X-ray emission from a hot corona around Sirius A. Preliminary calculations show that such a corona would need to be heated up to a few million K. The energy required would exceed the convective energy by nearly two orders of magnitude, even considering the fact that the anomalous silicon abundance[5] would yield an enhanced, soft X-ray line emission. It could be questioned whether the X-ray emission could be produced by accretion on to the white dwarf of a stellar wind from Sirius A. To generate an X-ray energy of, say, 10^{28} erg s⁻¹ (this a very conservative lower limit), the accretion rate $\dot{M}$ must be at least $10^{-15} M_\odot$ yr⁻¹ $(GM_B\dot{M}/R_B \approx 10^{28}; \; M_B = 1.05 \; M_\odot; R_B \approx 0.01 R_\odot$ (ref. 6), *G* is the gravitation constant). If it is assumed that the stellar wind speed is twice the escape velocity of Sirius A[7], then (with $M_A = 2.2 \; M_\odot$, and $R_A = 1.75 \; R_\odot$; ref. 5), the accretion efficiency $(M_B/M_A)^2 \; (R_A/8a)^2 \approx 3 \times 10^{-10}$. Thus, a rate of mass loss from Sirius A of more than $3 \times 10^{-6} M_\odot$ yr⁻¹ would be needed to produce a total X-ray luminosity of 10^{28} erg s⁻¹. This is about 10^3–10^6 times the expected mass loss for an A-type main-sequence star[8]. Even the mass loss from the A2 Ia supergiant α Cyg, estimated from the Mg II resonance lines[9], is only $\sim 3 \times 10^{-10} M_\odot$ yr⁻¹. Thus, the mechanism of accretion probably fails to explain the observed X radiation.

R. MEWE
J. HEISE
E. H. B. M. GRONENSCHILD
A. C. BRINKMAN
J. SCHRIJVER
A. J. F. DEN BOGGENDE

The Astronomical Institute,
Space Research Laboratory,
Beneluxlaan 21,
Utrecht, The Netherlands

1 Brinkman, A. C., Heise, J., and de Jager, C., *Philips tech. Rev.*, **34**, 43–59 (1974).
2 Den Boggende, A. J. F., *IEEE Trans. nucl. Sci.*, **22**, 595 (1975).
3 Den Boggende, A. J. F., and Lafleur, H. Th. J. A., *ibid.*, **22**, 555 (1975).
4 Catura, R. C., Acton, L. W., and Johnson, H. M., *Astrophys. J. Lett.*, **196**, L47–L49 (1975).
5 Latham, D. W., *Smithsonian Astrophysical Observatory Special Report 321* (1970).
6 Nariai, K., and Klinglesmith, D. A., *Proc. third Harvard–Smithsonian Conf. on Stellar Atmospheres* (Cambridge, Massachusetts, 1968).
7 Van den Heuvel, E. P. J., *Astrophys. J. Lett.*, **198**, L109–L112 (1975).
8 Lamers, H. J. G. L. M., thesis, Univ. Utrecht (1974).
9 Lamers, H. J. G. L. M., *Proc. R. Soc.* (in the press).

Anomalous temporal behaviour of Her X-1

DURING January 1975 the Leicester Sky Survey experiment aboard Ariel V observed anomalous X-ray emission the during off state of Her X-1. Figure 1*a* shows the positive observations preceded by an extended period when no radiation was detected, a 3σ limit being shown by horizontal bars. The counting rates are averages over an entire satellite orbit and arise from photons in the range 2.4 to 19.8 keV. The counting rate scale may be converted to Uhuru units for a typical Her X-1 spectrum by multiplying by 1.27. Figure 1*b* shows that about half of the following on state was observed but not the actual turn on. If the turn on had occurred at phase 0.2 in the first observed cycle, the midpoint of the anomalous emission would have preceded the turn on by 16.5 d. A turn on extrapolated from
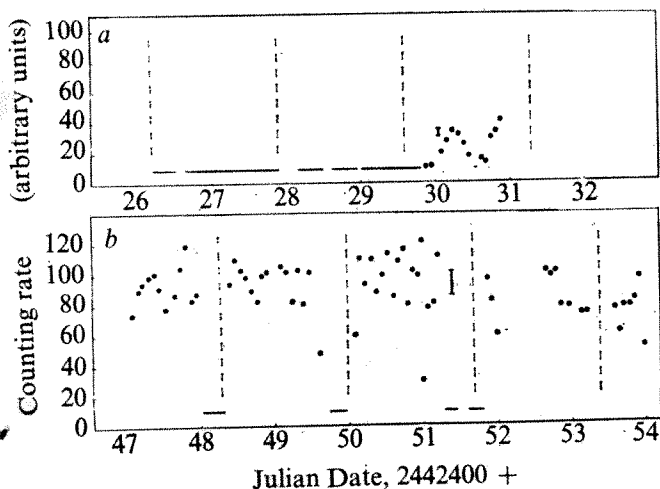
Fig. 1 Counting rate from Her X-1 during anomalous emission (a) and next regular on state (b). Vertical lines are mid-eclipse points, error bars are ±1σ random errors.

Copernicus data (A. C. Fabian, private communication), taken two 35-d cycles later, reduces this to 14 d.

Although Her X-1 was extensively studied with Uhuru, observations were concentrated on the on state so that the probability of detecting emission during the off state was low. The only other indication of low state emission[1] from Her X-1 also occurred approximately half way through the 35-d cycle and was estimated to have a strength of between 0.3 and 1.0 times the typical strength during the on-state. Also, the emission we observed showed considerable variation in intensity and when our observations ceased was at 0.4 of the average on state strength and still increasing.

The mechanism of mass transfer from primary star to the compact companion in binary X-ray source is crucial in determining the emission behaviour of the source on long time scales. The identification[2,3] of low mass star, HZ Herc, with the primary of the Her X-1 system suggests that the transfer is by Roche lobe overflow rather than by the stellar wind envisaged for the Krzeminski star/Cen X-3 system. In the former mechanism considerably more of the mass lost by the primary will be transferred to the secondary and the formation of the accretion disk is expected to be more orderly. Thus we anticipate that the long term temporal behaviour of Her X-1 will be simpler than that of, say, Cen X-3 where the density and ionisation state of the stellar wind have important roles in determining the activity and visibility of the X-ray emission[4].

The regularity of the extended low states of Her X-1 point to a clock-like mechanism with which the marching of the pre-eclipse dip seems to be connected (D. Gerend *et al.*, unpublished). These dips may be due to obscuration of the X-ray source by material at the point where the overflow hits the accretion disk[5] which itself may precess[6,7] and periodically prevent us seeing the still emitting source. This explanation may be contrasted with that involving 'wobble' of the neutron star which either periodically alters the direction of a sharply emitted X-ray beam[8] or actually causes the X-ray emission to be strongly modulated by giving rise to a geometrical mass gating action[9].

The presence of radiation from Her X-1 within the extended low places an additional constraint on these models, especially if the radiation could be shown to be a regular feature of the off state and to be always at a particular phase of it.

B. A. COOKE
C. G. PAGE

X-ray Astronomy Group,
Department of Physics,
University of Leicester, UK

1 Fabian, A. C., Pringle, J. E., and Rees, M. J., *Nature*, 224, 212 (1973).
2 Bahcall, J. N., and Bahcall, N. A., *Astrophy. J. Lett.*, 178, L1 (1972).
3 Forman, W., Jones, C. A., and Liller, W., *Astrophy. J. Lett.*, 177, L103 (1972).
4 Pounds, K. A., Cooke, B. A., Ricketts, M. J., and Turner, M. J., *Mon. Not. R. astr. Soc.*, 172 (in the press).
5 Pines, D., Pethick, C. J., and Lamb, F. K., *Proc. Sixth Texas Symp. Relativistic Astrophys. Ann. NY Acad, Sci.*, 224, 237 (1973).
6 Roberts, W. J., *Astrophys. J.*, 187, 575 (1974).
7 Katz, J. I., *Nature*, 246, 87 (1973).
8 Brecher, K., *Nature*, 239, 325 (1972).
9 Lamb, D. Q., Lamb, F. K., Pines, D., and Shaham, J., *Astrophys. J. Lett.*, 198, L21 (1975).

Whiskers and cosmic millimetre-wave sources

MANY extragalactic objects—N-type Galaxies, Seyferts and QSOs—are strong emitters of infrared and millimetre-wave radiation[1]. For Seyfert galaxies the most likely model for the 2–20 μm radiation involves a central source of primary radiation surrounded by a shell of solid particles, the dust absorbing the primary radiation and re-emitting it in the infrared[2]. There is independent evidence for the presence of dust in the nuclei of Seyferts including detections of differential reddening of [S II] lines[3]. The formation of dust could occur in the expansion phases following explosions of massive (or supermassive) stars[4] and the primary radiation may be in the form of ultraviolet photons, soft X rays or low energy cosmic-ray nucleons[5].

Dust particles invoked for these models have so far been assumed to be similar to solid particles which are deduced to exist in our own Galaxy. Nearly spherical grains with radii in the range 10^{-6}–10^{-5} cm could account for the observed 2–20 μm fluxes with reasonable mass requirements. A serious difficulty arises with regard to total grain mass if the same grains are to explain the observed millimetre and sub-millimetre wave radiation simply because of the low antenna efficiencies of very small particles at long wavelengths.

The total millimetre-wave fluxes of extragalactic sources and the peak wavelengths are somewhat uncertain. For Seyfert galaxies the average energy output at millimetre wavelengths is $\sim 3 \times 10^{44}$ erg s^{-1} and for QSOs a range 10^{44}–10^{46} erg s^{-1} is likely, the spectra probably peaking at $\lambda \sim 120$ μm. This value of the peak wavelength indicates a minimum grain temperature $T_g \sim 30$–50 K (depending on the wavelength dependence of the emissive efficiency). If T_g is the grain temperature at the outer edge of the shell, the flux in the range λ, $\lambda + d\lambda$ is

$$F_\lambda d\lambda \simeq \kappa_{abs}(\lambda) M_g \pi B_\lambda(T_g) d\lambda \tag{1}$$

where M_g is the total grain mass, $\kappa_{abs}(\lambda)$ is the mass absorption coefficient of grains, and $B_\lambda(T_g)$ is the Planck function. The maximum absorption efficiency of a small spherical particle being[6]

$$Q_{abs}^{max} \sim 10a/\lambda \tag{2}$$

in a typical case we find

$$\kappa_{abs}^{(sph)}(\lambda) \lesssim 30/4\lambda s \tag{3}$$

so that, taking $s = 2.2$ g cm^{-3},

$$F_\lambda d\lambda \lesssim 1.7 \times 10^{29} (T_g/\lambda^5) d\lambda \ (M_g/M_\odot) \text{ erg s}^{-1}$$

using equation (1) and the Rayleigh–Jeans approximation for $B_\lambda(T_g)$.

Taking $T_g \simeq 30$ K as a representative value, and setting $\lambda \simeq \Delta\lambda \simeq 1$ mm, we obtain

$$F_\lambda \Delta\lambda < 5 \times 10^{34}(M/M_\odot) \text{ erg s}^{-1} \tag{4}$$

for spherical particles. Thus, to explain a millimetre-wave output of 3×10^{41} erg s^{-1} for a Seyfert nucleus we require grain masses $> 5 \times 10^6 \ M_\odot$; and for $F_\lambda d\lambda \simeq 10^{44}$–$10^{46}$ erg s^{-1}, in the case of QSOs, we require grain masses exceeding 2×10^9–$2 \times 10^{11} M_\odot$. These grain masses must be regarded as untenably large.

These mass estimates are significantly reduced if long whisker grains of lengths 10^{-2}–10^{-1} cm are present in the emitting dust shell. For cylinders with length $l \gg a$, and at wavelengths $\lambda \leqslant l$ the mean mass absorption cross section for randomly oriented particles is approximately[7]

$$C_{abs}(\lambda) = \frac{4\pi a^2 l \sigma(\lambda)}{3c}\left\{1 + \frac{8K}{(K+1)^2 + 4(\sigma\lambda/c)^2}\right\} \qquad (5)$$

where $\sigma(\lambda)$ is the optical conductivity, K is the dielectric constant, and a is the cross-sectional radius. The second term is curly brackets is in general negligible compared with the first, and for graphite, where optical data are available up to millimetric wavelengths[8] $\sigma(1\,\text{mm}) \simeq 10^{15}\,\text{s}^{-1}$. Thus equation (5) gives a mass absorption coefficient

$$\kappa_{abs}^{(cyl)}(\lambda) \simeq 4\pi\sigma(\lambda)/3cs \qquad (6)$$

where s is the specific gravity of grain material, and using equation (3) we have

$$\frac{\kappa_{abs}^{(cyl)}(\lambda)}{\kappa_{abs}^{(sph)}(\lambda)} > \frac{4\pi\sigma/3cs}{15/2\lambda s}$$

$$\simeq 1.5(\sigma/10^{15})\,(\lambda/10^{-4}) \qquad (7)$$

For graphite with $\sigma(1\,\text{mm}) \simeq 10^{15}\,\text{s}^{-1}$, $\lambda \sim 10^{-1}$ cm we find the required mass estimates in the form of whisker grains are down by a factor $\sim 1.5 \times 10^3$. This gives grain mass estimates of $10^3 M_\odot$ for explaining the millimetre fluxes from Seyferts, and 10^6–$10^8 M_\odot$ for QSOs. The implied hydrogen masses $10^5 M_\odot$–$10^{10} M_\odot$ are now within the permissible range. Impure ice whiskers are expected to have a similar value of low frequency conductivity, so these mass estimates are also appropriate for ice whiskers, or formaldehyde polymer whiskers coated with impure ice.

The growth of formaldehyde whiskers in interstellar space has already been discussed by one of us [9,10]. Crystal growth in whisker form is also well known to occur in the case of graphite condensing in suitable laboratory conditions[11,12]. We will discuss the whisker growth process in greater detail elsewhere.

It may be argued that either formaldehyde polymers or graphite whisker growth could take place in conditions prevailing near the nuclei of Seyferts and QSOs. The former case arises where the ambient C/O ratio exceeds unity, and the latter where O/C exceeds unity. In the latter case, the oxygen uncondensed as H_2CO may be deposited as H_2O mantles on the formaldehyde whiskers. The maximum length l of whisker grains is determined by the condition for the exhaustion of condensable gas phase atoms in most cases of interest and is given by

$$l = (n_g/n_H)^{-1}(\pi a^2)^{-1}s^{-1}fm_A \qquad (8)$$

Here f is the fraction of condensable atoms (by numbers) relative to H, m_A is their mass per molecule, a is the radius of the grains (or base sites on grains) which act as condensation nuclei, and s is the specific gravity of the condensing solid. For interstellar type grains with $a \simeq 10^{-5}$ cm, $n_s/n_H \sim 10^{-12}$, $s = 2.5$, $f \sim 10^{-3}$, $m_C = 12m_H$ we find $l \lesssim 1\,\mu\text{m}$. Much longer particles could grow in the vicinity of galactic nuclei if lower n_g/n_H ratios and/or lower values of a are appropriate. Such situations could arise due to sputtering or evaporation of original grain nuclei in an explosive phenomenon, or simply from a low starting value of n_g/n_H. Grain lengths of 100 μm–1 mm required for our radiation model may thus be justified fairly easily.

The whisker growth process is extremely rapid, and could easily occur within the time scales of protostellar collapse. The existence of whisker grains in galactic protostellar clouds would allow extremely bright far-infrared sources with very low dust masses. For example, the millimetre emission from OMC1

could be explained with whisker grain masses of less than $0.02 M_\odot$.

M. G. EDMUNDS
N. C. WICKRAMASINGHE

Department of Applied Mathematics and Astronomy,
University College,
PO Box 78, Cardiff CF1 1XL, UK

1 Rowan-Robinson, M., in *Proc. 2nd Euro. Meeting Int. astr. Union, 1974* (edit. by Muller, E.), (in the press).
2 Rees, M. J., Silk, J. I., Werner, M. W., and Wickramasinghe, N. C., *Nature*, 223, 788 (1969).
3 Wampler, E., *Astrophys. J.*, 164, 1 (1971).
4 Hoyle, F., and Wickramasinghe, N. C., *Nature*, 218, 1126 (1968).
5 Wickramasinghe, N. C., *Nature*, 230, 166 (1971).
6 Hoyle, F., and Wickramasinghe, N. C., *Nature*, 223, 459 (1969).
7 Wickramasinghe, N. C., *Light Scattering Functions for Small Particles with Applications in Astronomy* (Adam Hilger, London, 1963).
8 Taft, E. A., and Phillipp, H. R., *Phys. Rev.*, 138A, 197 (1965).
9 Wickramasinghe, N. C., *Mon. Not. R. astr. Soc.*, 170, 11p, (1975).
10 Wickramasinghe, N. C., *Nature*, 252, 462 (1974).
11 Bacon, R., and Bowman, J. C., *J. appl. Phys.*, 28, 826 (1957).
12 Bacon, R. *Cambridge Conference on Strength of Whiskers and Thin Films* (Cambridge University Press, Cambridge 1958).

Water in non-carbonaceous stony meteorites

WE report here on high-voltage electron microscope observations of the hydrous alteration products of olivine in an achondrite and an ordinary chondrite—two classes of meteorite in which hydrous minerals are rare. We argue that the Nakhla achondrite, and perhaps the Weston chondrite, contain water of extraterrestrial origin which was mobilised by mild shock deformation. The observations suggest that both meteorites have unusual volatile constituents.

Hydrous minerals are rare in meteorites other than types I and II carbonaceous chondrites[1]. It has been pointed out that iddingsite occurs in the 'diopside'-olivine Nakhla achondrite (T. E. Bunch and A. M. Reid, 1974 Meeting of the Meteoritical Society). Iddingsite is a disequilibrium mixture of products of the hydrothermal alteration of olivine, in which approximate hexagonal close packing of the oxygen ions is retained[2]. In the Nakhla achondrite it occurs mainly as red-brown veins in the large olivine grains. Using transmission electron microscopy we have examined a vein in material prepared by ion thinning[3]. The vein was found to have been formed as a result of alteration along a crack, which in turn is attributable to shock deformation since the adjacent olivine shows mosaicism and dislocation substructures characteristic of mild shock effects[4]. The fine-grained alteration product becomes colloidal towards the centre of the vein (Fig. 1a). The association with shock deformation supports the idea that the iddingsite is of extraterrestrial origin. The Nakhla achondrite is essentially an igneous rock which formed from a relatively oxidised melt, producing Fe^{+2}-rich silicates and primary magnetite; magmatic water is likely to have been present during its formation. The evidence for later mobilisation of the water by mild shock suggests that here leaching of rubidium may have been responsible for the radiometric anomalies[5,6] in which whole-rock specimens deviate from the 1.24 Gyr mineral isochron believed to represent the crystallisation age[5]. The presence of water suggests that the parent body was not outgassed earlier than 1.24 Gyr BP.

The Weston chondrite has similar iddingsite veins in olivine. Where olivine with only slight mosaicism is crossed by narrow veins (less than 1 μm thick), electron diffraction patterns were obtained showing a topotactic transition to a goethite-like condition, similar to that described in terrestrial iddingsite[2]. In the fine-grained matrix of the meteorite, we also found abundant iddingsite and similar material filling interstices. This accounts for the high content of bound water (0.98% by weight), in the bulk chemistry[7]. In Fig. 1b the relatively large crystal is olivine, with a rim of fine material which, by comparison with the vein occurrences, may be described as iddingsite. This material cements the grains in Fig. 1b. We found that the
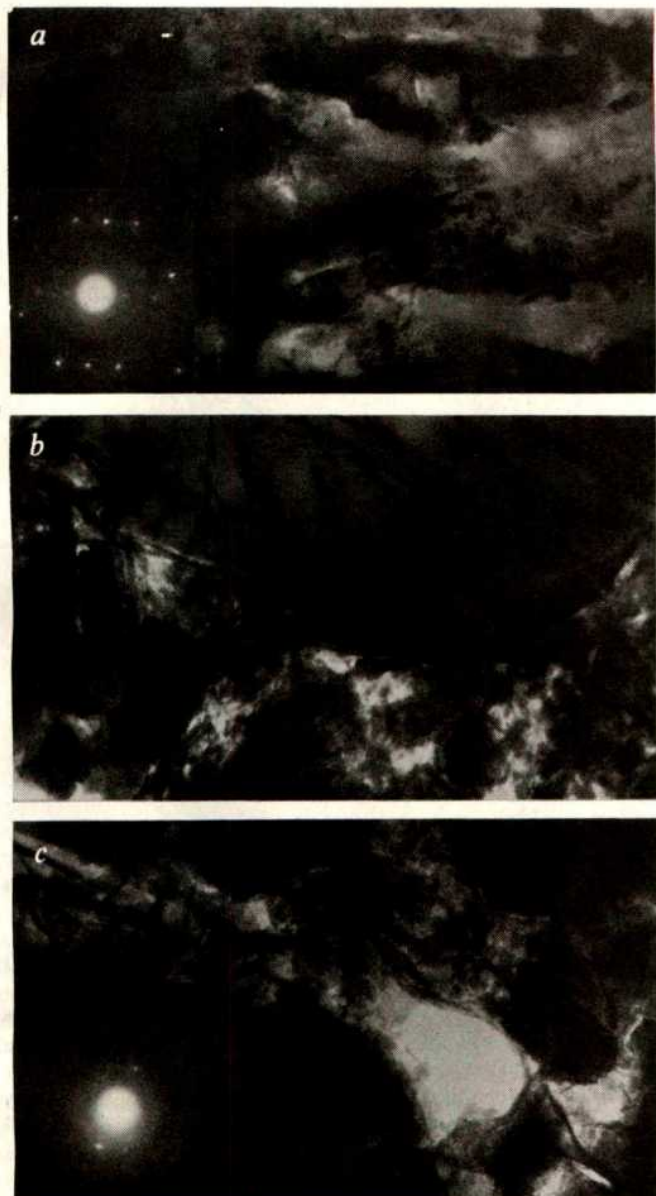
Fig. 1 High-voltage (1 MV) electron micrographs showing meteoritic iddingsite. Insets are diffraction patterns from the fine-grained areas. *a*, The Nakhla achondrite (BM 1913,26) × 18,000—diffraction pattern comprises spots from olivine and rings from the alteration product; *b* and *c*, the Weston chondrite (BM 1920,349)—*b*, ×13,000; *c*, ×16,400.

alteration makes an important contribution to the mechanical cohesion of the Weston chondrite. Where relatively coarse, the material often has a morphology comprising matted laths (Fig. 1*c*), and gave diffraction patterns dominated by rings corresponding to lattice spacings of 2.46 ± 0.04 and 1.49 ± 0.05Å. Where strong, they were accompanied by other rings, consistent with patterns from randomly oriented crystallites of a clay phyllosilicate with $a \approx 5.2$ Å. Smaller grains tended to have equidimensional images, and diffraction gave pseudohexagonal arrays of diffuse spots comparable with basal diffraction patterns of the disordered phyllosilicate constituent in terrestrial iddingsite[2].

The Weston chondrite is mildly shock-lithified (J. R. A. and D. J. Barber, unpublished). If the water was present during the shock events, it would have been mobilised and could thus have produced the observed alteration. The possibility that the hydration occurred later should, however, be considered. The Weston chondrite (like the Nakhla achondrite) is an observed fall and, therefore, has not suffered normal terrestrial weathering, but it is reduced and porous, like the Apollo 16 'rusty rocks' in which hydrous minerals are thought to have been produced through the hydrolysis of primary lawrencite, $FeCl_2$, by terrestrial atmospheric water vapour[8]. The Weston chondrite contains, however, an order of magnitude more water than do the Apollo 16 rocks, and to attribute this to the lawrencite reaction requires an exceptionally high initial content of the volatile element chlorine; abnormal enrichment of extraterrestrial volatiles is found in the Apollo 16 rocks themselves[8].

Weston is a 'gas-rich' chondrite[9], which means that some individual grains suffered unshielded irradiation by the solar wind, resulting in the implantation mainly of hydrogen and helium. The observed alteration cannot, however, be attributed to the formation of water by reaction between mineral grains and solar wind ions, as lunar surface breccias are generally anhydrous. If aggregation of the grains occurred on a small atmosphere-free body[10,11], a magmatic source of reactive volatile elements is also unlikely. On the other hand, a likely source is a 'dust' component of type I and/or type II carbonaceous chondrite composition. Macroscopic inclusions of carbonaceous chondrite are certainly present in some gas-rich meteorites[10]. These meteorites probably formed during the terminal stages of accretion of their parent bodies, in an environment that was favourable for the incorporation of such inclusions[10,11]. Moreover, a carbonaceous chondrite component would carry water, because it would consist largely of hydrous phyllosilicates[1]. Because the hydrous minerals in the Weston chondrite were found on cracks and grain boundaries, it is unlikely that they could be unaltered grains from a carbonaceous chondrite source though such a source could have supplied the water for the subsequent alteration. If all the water in the Weston chondrite is from this source, the indicated minimum content of type I carbonaceous chondrite is 5% by weight, using the figure of 20% for the water content of type I material[1]; for type II, the corresponding minimum content in Weston is 7.5% by weight. These are minimum figures because water may have been lost during accretion. If the Weston chondrite does contain more than 5% of a carbonaceous chondrite component, it should be identifiable chemically. Neither the major element data[7] nor the Kr and Xe isotopic abundances[12] are, however, diagnostic of its presence. An attempt is being made to characterise any unusual component which may be present in the Weston chondrite. Until this has been done, the additional possibility that the Weston chondrite is from a body which had a hydrous atmosphere must also be left open. Whatever the source of the water, the pervasive alteration suggests that the matrix of the Weston chondrite incorporated an unusually large amount of chemically reactive material.

J. R. ASHWORTH

Department of Physics,
University of Essex,
Wivenhoe Park,
Colchester CO4 3SQ, UK

R. HUTCHISON

Department of Mineralogy,
British Museum (Natural History),
London SW7 5BD, UK

1 Mason, B., *Meteoritics*, **7**, 309–326 (1972).
2 Gay, P., and LeMaitre, R. W., *Am. Miner.*, **46**, 92–111 (1961).
3 Barber, D. J., *J. mater. Sci.*, **5**, 1–8 (1970).
4 Ashworth, J. R., and Barber, D. J., *Earth planet. Sci. Lett.* (in the press).
5 Gale, N. H., Arden, J., and Hutchison, R., *Earth planet. Sci. Lett.*, **26**, 195–206 (1975).
6 Papanastassiou, D. A., and Wasserburg, G. J., *Geophys. Res. Lett.*, **1**, 23–26 (1974).
7 Mason, B., and Wiik, H. B., *Am. Mus. Novit.*, **2220** (1965).
8 Epstein, S., and Taylor, H. P., Jr, *Proc. fifth lunar Sci. Conf., Geochim. cosmochim. Acta Suppl.*, **5** (2), 1839–1854 (1974).
9 Schultz, L., Signer, P., Lorin, J. C., and Pellas, P., *Earth planet. Sci. Lett.*, **15**, 403–410 (1972).
10 Wilkening, L. L., *Geochim. cosmochim. Acta*, **37**, 1985–1989 (1973).
11 Pellas, P., in *From Plasma to Planet (Nobel Symposium* 21) (edit. by Elvius, A.), 65–92 (Almqvist and Wiksell, Stockholm, 1972).
12 Eugster, O., Eberhardt, P., and Geiss, J., *J. geophys. Res.*, **74**, 3874–3896 (1969).

Lag times for oceanic responses to climatic change

RECENT studies of deep-sea sediment cores have defined changes in surface water circulation[1], in continental ice volume as recorded by oxygen isotope variations in foraminiferal tests[2], and in the corrosiveness of near-bottom water to calcium carbonate[3,4] during the past million years. These changes are all attributed to the major climatic changes that have occurred during the Quaternary[1,2,5,6].

Few of the studies have attempted to establish whether the various changes are synchronous, or whether there are leads and lags in the system[7]. Such information is critical in evaluating theories and models of climatic change, and in sorting out causes from effects. Thus, if bottom water changes precede surface water and ice volume changes during deglaciation, models like that of Newell[8] are favoured, whereas if the ice volume decreased first, an albedo-related cause is more likely.

To determine sequences of change, either extremely accurate time scales for the records of the oceanic system and the continental ice volume, or records of the two systems from the same core samples are needed. The latter approach eliminates problems of correlation or inaccuracies of time scales in different deep-sea cores, and is the approach used here.

The sediment core selected for detailed study (core Y69-106P, 2°59′N, 86°33′W, 2,870 m) was taken in the eastern equatorial Pacific, an area where the surface circulation varied significantly from glacial to interglacial times[9]. To establish a time scale throughout the core, a sedimentation rate model was constructed assuming that the rate of accumulation of quartz has been constant at the core location (a reasonable assumption given the depositional regime in the area[10]). The fact that calculated accumulation rates of quartz were constant to within 3% between points dated by three carbon-14 determinations, an $^{40}Ar/^{39}Ar$ age, and oxygen isotope and biostratigraphic boundaries of known ages, supports this assumption. Details of the model are given in ref. 10. Measurements of carbonate, opal, and quartz concentrations together with bulk density measurements were transformed by the model to give absolute accumulation rates (g cm^{-2} 10^{-3} yr) for the total sediment, calcium carbonate, opaline silica, and the remaining (non-biogenic, non-quartz) detritus during the past 450,000 yr.

Conversion of the raw calcium carbonate analyses to accumulation rates eliminates the problems inherent in working with concentration data. In the Pacific, changes in the concentration of calcium carbonate probably reflect changes in accumulation rate of carbonate particles, but in the Atlantic, fluctuations in

Fig. 1 Calcium carbonate and opaline silica accumulation rates (in g cm^{-2} 10^{-3} yr) and oxygen isotopic data from core Y69-106P.
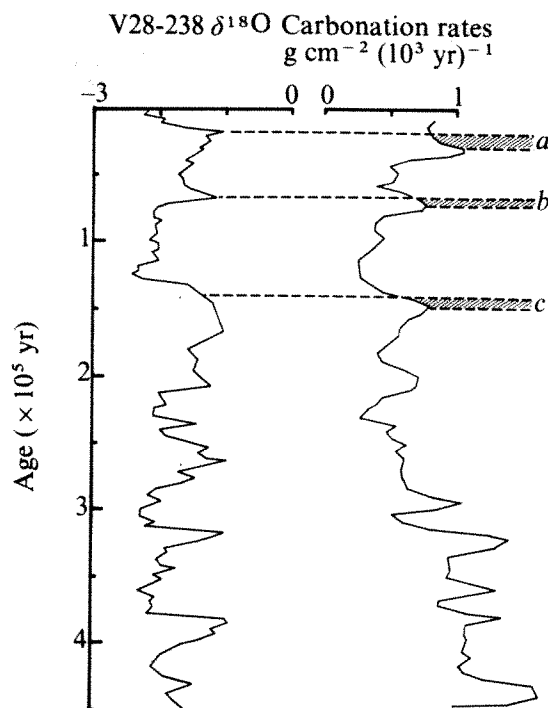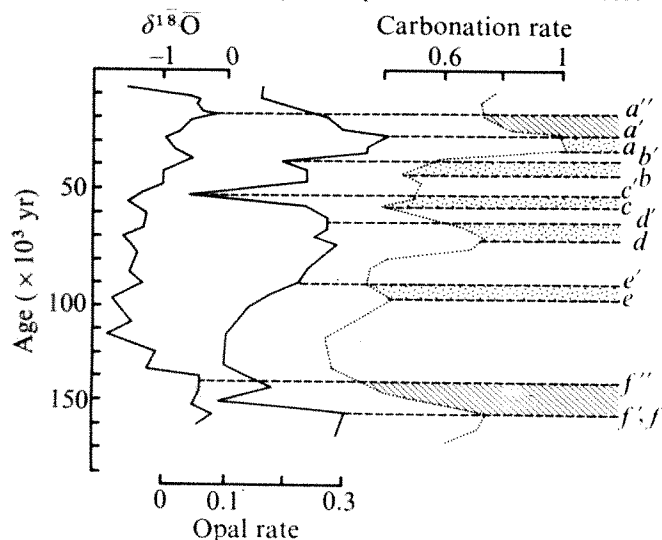

Fig. 2 Calcium carbonate accumulation rate in core Y69-106P compared to oxygen isotopic analyses from core V28-238.

carbonate concentration are strongly influenced by variable dilution as a result of changes in the accumulation rate of terrigenous material[11].

Comparison of the silica and carbonate accumulation rate curves and the oxygen isotopic composition of foraminiferal calcite in Y69-106P (Fig. 1) suggests that climatic and oceanographic changes have not been synchronous. The accumulation rate curves for opaline silica and calcium carbonate during the past 150,000 yr are very similar (Fig. 1), but there seems to be a slight lag between the two curves during this period, changes in calcium carbonate accumulation preceding changes in opal accumulation rates. The calcium carbonate and opaline silica accumulation rates seem to be responding to the same changes in the driving mechanism, presumably climate, but the response times are different. The average lag time between change in carbonate and opal accumulation rate (Fig. 1a–f) is 2,600 yr.

The relation between the isotopic data and the other parameters is less apparent, but again the carbonate rate curve appears to lead, this time by 5,000–10,000 yr. Confirmation of this lead is provided by a comparison of the carbonate curve of Y69-106P with a more complete isotopic record from western Pacific core[2] V28-238 (Fig. 2). For the last three glacial maxima, the carbonate peak precedes the isotopic (ice volume) peak by about 5,600 yr.

Opal accumulation rates seem to be controlled by biological productivity in the surface waters[10,12] whereas changes in the carbonate accumulation rates reflect changes in the dissolution of calcium carbonate by bottom waters[3]. Thus the lags between changes in the calcium carbonate accumulation rates, the opaline silica accumulation rates, and the oxygen isotope analyses from core Y69-106P indicate that the following sequence of events occurred in the eastern equatorial Pacific during the transition from the most recent glacial to the present interglacial period (Fig. 1, a, a′, a″):

An increase in corrosiveness of the bottom water (indicated by a reduction in calcium carbonate accumulation rates), preceded a decrease in surface productivity (indicated by a reduction in opal accumulation rates), which in turn preceded the recession of continental glaciers (indicated by a reduction in the ^{18}O content of foraminiferal carbonate). The period covered by this sequence of events was about 5,600 yr with

about 2,600 yr (an average of events *a–f* Fig. 1) elapsing between the bottom water and surface circulation changes and 3,000 yr between the change in surface circulation and the recession of the continental glaciers. Because of the relatively wide spacing in time of individual samples from Y69-106P (the average sample interval is about 4,000 yr) these estimates represent maximum lag times.

If the lags between the carbonate and opal accumulation rate curves were random and independent, the probability of the observed succession in the six events *a–f* in Fig. 1 would be 0.016—highly unlikely. Rather, the observed changes seem to be systematic and are consistent with two recent models[8,13] of climatic change. Newell[8] has suggested that the termination of a glacial period is first marked by a decrease in the rate of formation or even stagnation of bottom waters (this would result in an increase in dissolved carbon dioxide in the bottom waters, leading to accelerated carbonate dissolution). He further suggests that the bottom water stagnation would be succeeded by reduced upwelling (leading to reduced opal deposition) and finally by wasting of continental glaciers. The data from core Y69-106P are in complete accord with Newell's model. If confirmed by data from other geographical areas, these results support continued development of the model and seem capable of providing accurate estimates of the lag times involved in the change of the oceanic and cryospheric systems from glacial to interglacial modes.

NICKLAS G. PISIAS
G. ROSS HEATH
TED C. MOORE, JR

Graduate School of Oceanography,
University of Rhode Island,
Kingston, Rhode Island 02881

1 McIntyre, A., Ruddiman, W. F., and Jantzen, R., *Deep-Sea Res.*, **19**, 61-77 (1972).
2 Shackleton N. J., and Opdyke, N., *Quat. Res.*, **3**, 39-55 (1973).
3 Berger, W. H., *J. foram. Res.*, **3**, 187-195 (1973).
4 Thompson, P. R., and Saito, T., *Geology*, **2**, 333-338 (1974).
5 Arrhenius, G., *Swedish Deep-sea Expeditions* (1947-1948) *Reports*, **5**, 89 p. (1952).
6 Hays, J. D., Saito, T., Opdyke, N. D., and Burckle, L. H., *Geol. Soc. Am. Bull.*, **80**, 1481-1514 (1969).
7 Luz, B. and Shackleton, N. J., *Cushman Found. Foram. Res. Spec. Pub.*, **13**, 142-150 (1974).
8 Newell, R. E., *Quat. Res.*, **4**, 117-127 (1974).
9 Molina-Cruz, A., thesis, Oregon State Univ. (1975).
10 Pisias, N. G., with appendix by Shackleton, N. J., *Geol. Soc. Am. Spec. Pap.* (in the press).
11 Broecker, W. S., Turekian, K. K., and Heezen, B. C., *Am. J. Sci.*, **256**, 503-517 (1958).
12 Heath, G. R., *Paleo-oceanography: SEPM Special Pub.*, **20**, 77-93 (1974).
13 Weyl, P. K., *Meteorol. Monogr.*, **8**, 37-62 (1968).

Strain resulting from adhesive action of water in capillary bridges

WE report here on the measurement of adhesive forces of thin films of water between glass plates. Our approach differed

Fig. 1   Displacement, d*b*, in a deformable isolated capillary. (From surface-energy considerations.)
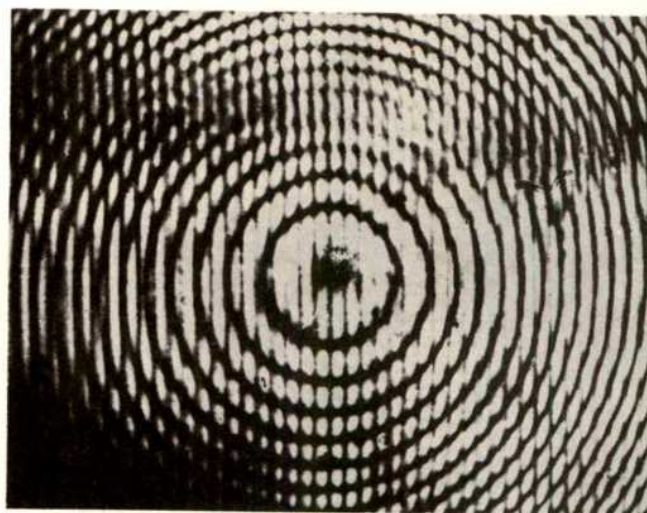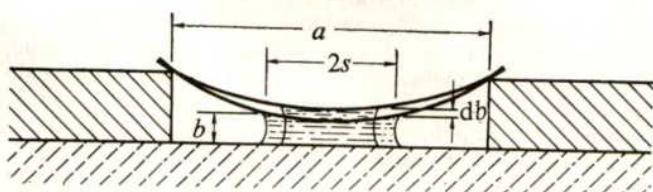

**Fig. 2**   Interference fringe pattern obtained by a mechanical loading of 20,482 dyne.

from the traditional method of measuring the force necessary to rupture the liquid film in tension[1-3]; we used interferometry to measure the elastic strains produced around the bridges. The main pupose of the study was to assess the degree of agreement between measured adhesive forces and those predicted by classical capillary theory.

When a surface-energy consideration is applied to a liquid bridge between a deformable capillary wall and a rigid capillary wall, the work done by the force F during an increase, d*b*, in the distance b (Fig. 1) is equal to the energy of wetting and the elastic bending energy of the capillary wall:

$$F\mathrm{d}b = (\gamma_{SV} - \gamma_{SL})\mathrm{d}A_{SL} + \gamma_{LV}\,\mathrm{d}A_{LV} + \mathrm{d}U \qquad (1)$$

where γ is an interfacial energy, A an interfacial area, and the subscripts S, V and L denote solid, vapour and liquid, respec-

Fig. 3   Interference fringe pattern obtained by capillary adhesion. An adhesive force of 9,510 dyne is measured.
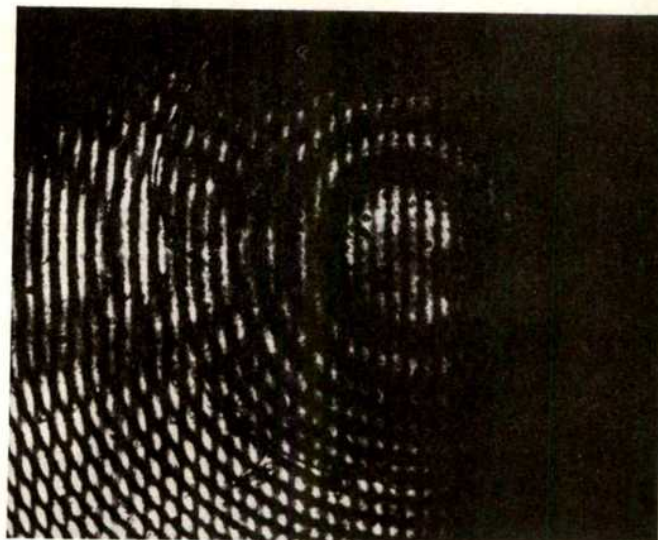
tively; U is the bending energy in the capillary wall. From the Young–Dupre equation, $\gamma_{LV}\cos\theta$ can be substituted for $(\gamma_{SV} - \gamma_{SL})$ with θ being the contact angle of the liquid on the

capillary wall. The theory of bending of plates[4] gives:

$$dU = Fdb/\cos(s\pi/a) \qquad (2)$$

When $s \gg b$, the term $\gamma_{LV}dA_{LV}$ is negligible, and by using β to represent $s\pi/a$ equations (1) and (2) can be combined to give

$$\frac{F}{\pi s^2}(1 + \frac{1}{\cos\beta}) = \gamma \cos\theta\frac{2}{b}, \qquad (3)$$

which indicates a linear relationship with a slope of $\gamma \cos\theta$.

A helium–neon laser beam was directed normal to two parallel glass capillary walls separated by a metal spacer 2 μm thick. Deflections of the deformable wall under applied forces gave rise to interference fringe patterns which are linearly related to the magnitudes of the applied forces. Mechanical loading was used to produce fringe patterns for calibration (Fig. 2).

Fringe patterns were obtained with water bridges having diameters of 0.04, 0.070, 0.12 and 0.13 cm (Fig. 3). Next, the forces of adhesion were calculated based on the calibration.
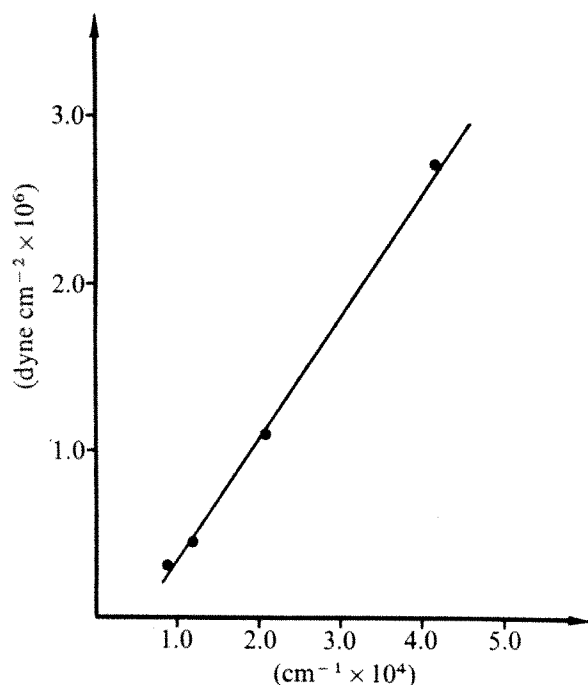
Fig. 4 The relationship of $(F/\pi s^2)(1 + 1/\cos\beta)$ (ordinate) and $2/b$ (abscissa) for a deformable glass–water capillary system. The experimental value of $\gamma \cos\theta$ is 73.9 dyne cm^{-1}, compared with a predicted value of 72 dyne cm^{-1}.

Using these results, Fig. 4 was plotted. The slope of the linear regression line is 73.9 dyne cm^{-1} which, from equation (3), would be equal to γ since θ was near zero. This close agreement with the surface tension value of water supports the correctness of equation (3). The results indicate that the surface energy of wetting can account for the elastic strains observed.

P. L. FAN
W. J. O'BRIEN

University of Michigan,
Ann Arbor, Michigan 48104

[1] McFarlane, J. S., and Tabor, D., *Proc. R. Soc.*, A202, 224–243 (1950).
[2] O'Brien, W. J., and Yu, C. U., *Surf. Sci.*, 32, 739–742 (1972).
[3] O'Brien, W. J., and Hermann, J. J., *J. Adhes.*, 5, 91–103 (1973).
[4] Timoshenko, S., *Theory of Elastic Stability*, 314–316 (McGraw-Hill, New York, 1936).

Three-coordinated magnesium in dehydrated offretite

OFFRETITE is a rare natural zeolite whose synthetic equivalent, zeolite O, is commercially important as a molecular sieve[1]. Its aluminosilicate framework is topologically related to that of erionite[2], an abundant natural zeolite[3]. The catalytic properties of molecular sieve zeolites have been ascribed to framework-bound protons, strong electrostatic fields, and oxygen vacancies in the framework[4]. As befits the small radius of Mg^{2+} ions (0.049 nm for four-coordination, 0.072 for six-coordination and 0.089 for eight-coordination)[5], Mg normally occurs in six-coordination with oxygen and hydroxyl, though four, five and eight-coordinations occur infrequently (for example, four-akermanite: $Ca_2MgSi_2O_7$, Mg–O = 0.1876 nm; five-grandidierite: $(Mg,Fe)Al_3O.BO_4.SO_4$, 0.2176, 0.2054, 0.2040, 2×0.1970; farringtonite: Mg_3PO_4, 0.196, 0.197, 0.201, 0.206, 0.214; eight-pyrope: $Mg_3Al_2Si_3O_{12}$, 4×0.2196, 4×0.2342)[6]. We report here on the occurrence of three-coordinated Mg in dehydrated offretite, which should produce a strong electrostatic effect on adjacent sorbed molecules.

Table 1 Atomic coordinates

	x/a	y/b	z/c
Si(1)*	0.9998(3)	0.2277(2)	0.2177(2)
Si(2)	0.0968(3)	0.4273(3)	0.5
O(1)	0.0408(6)	0.3511(5)	0.3182(9)
O(2)	0.0896(3)	0.9104(3)	0.2783(10)
O(3)	0.8722(9)	0.1278(4)	0.2879(11)
O(4)	0.0030(9)	0.2483(7)	0
O(5)	0.2425(6)	0.7575(6)	0.5
O(6)	0.4592(7)	0.5408(7)	0.5
Ca	0	0	0.5
K	0.4964(12)	0.5036(12)	0
Mg	0.333	0.667	0.5

*Numbers in brackets are atom designation numbers.

Hydrated offretite[7] $(K_{1.1}Ca_{1.1}Mg_{0.7}Si_{12.8}Al_{5.2}O_{36}.15H_2O)$ contains a K ion in each cancrinite cage, a pentahydrate of Mg in each gmelinite cage, and hydrated Ca ions in the main channels. Electron microprobe analysis of a new crystal, from Mount Simiouse, France, showed that this had $K_{1.04}Ca_{1.04}Mg_{0.95}Al_{5.2}Si_{12.8}O_{36}$ atoms in each cell. The crystal was dehydrated in a silica capillary for 22 h at 500 °C and 10^{-5} torr before sealing. Using MoKα radiation, 4,700 intensity measurements at room temperature yielded 514 independent non-zero diffractions in P6̄m2, a 1.3229(5), c 0.7338(4) nm. Least-squares refinement yielded the coordinates shown in Table 1.

The stereo plot (Fig. 1) shows Mg occupying the centre of a single six-ring where it is bonded to three oxygens at 0.208(1) nm. The six-ring is very distorted to accommodate this short distance and the other three oxygens are displaced outwards to 0.288(2) nm. Some weak bonding may occur to these three oxygens, but the strong disparity in the distances implies that the Mg is effectively three-coordinated. Each Ca ion lies between two six-rings which are distorted so that three oxygens from each give near-octahedral coordination at 0.2619(8) nm. Each K ion lies at the middle of a distorted eight-ring with four oxygens at 0.3258(7) as nearest neighbours and two more at 0.3350(6) nm. All the cation–oxygen distances are longer than the values expected from simple ionic theory and distances found in most silicates (for instance, Ca–O = 0.24; K–O = 0.27; and predicted Mg–O for three-coordination is less than 0.19 nm). The mean Al, Si–O distance of 0.1631 nm is less than the value of 0.165 obtained by interpolating the Al/Si ratio of offretite between the data for anhydrous framework silicates[8]. This suggests that the bonding in the framework of the dehydrated zeolite is stronger than in normal silicates and that the bonding
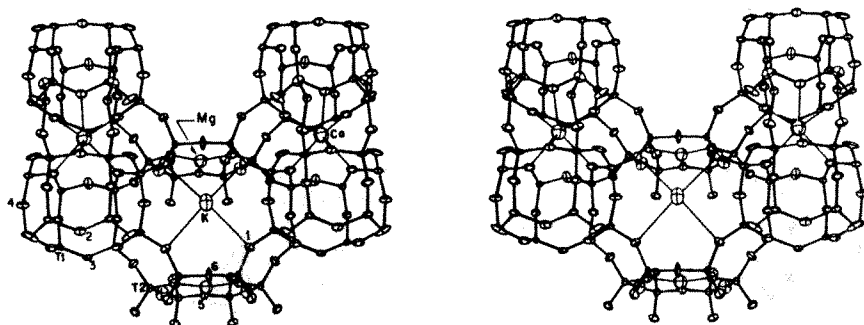
Fig. 1 Stereoplot of dehydrated offretite. Ellipses drawn at 30% probability level.

between extraframework cations and framework oxygens is weaker.

The Mg ion of dehydrated offretite should have a strong electrostatic field around it, and sorbed molecules should be strongly attracted to form a complex. Perhaps catalytic properties would result. In the dehydrated structure[9] of mazzite, which is another zeolite, Mg was tetrahedrally coordinated to three oxygens of a six-ring plus a fourth oxygen species which might be either a water molecule or a hydroxyl ion. Perhaps addition of one water molecule to each Mg ion would result in a similar coordination in offretite. Disproportionation of a water molecule into a hydroxyl ion and a proton could occur, with the latter condensing with a framework oxygen to yield a framework hydroxyl. The latter might be a Brønsted-acid catalyst (see, for example, ref. 4).

The unusual coordination of Mg in dehydrated offretite results from the spatial restrictions posed by the topology and geometry of the aluminosilicate framework. The four-rings cannot accommodate the Mg ion because of electrostatic repulsion from the Al,Si ions, and the centre of a six-ring offers the best of a set of bad options. Because of spatial restrictions the six-ring cannot pucker sufficiently to yield a near octahedral coordination, though it can pucker to give a triangular coordination. Thus the Mg ion, which would have had a reasonable coordination in the hydrated zeolite, becomes forced into an unusual coordination. A similar situation occurs in dehydrated Type A zeolites, in which transition metal ions have been found in three-coordination at or near the centre of six-rings[10,11].

Full details, including the nature of an erionite intergrowth, will be published elsewhere.

W. J. MORTIER
J. J. PLUTH
J. V. SMITH

Department of Geophysical Sciences,
University of Chicago,
Chicago, Illinois 60637

1 Breck, D. W., *Zeolite Molecular Sieves* (Wiley, New York, 1974).
2 Gard, J. A., and Tait, J. M., *Adv. Chem. Ser.*, **101**, 230 (1971).
3 Munson, R. A., and Sheppard, R. A., *Min. Sci. Eng.*, **6**, 19 (1974).
4 Rabo, J. A., *Zeolite Chemistry and Catalysis* (Am. chem. Soc., Washington, in the press).
5 Shannon, R. D., and Prewitt, C. T., *Acta crystallogr.*, **B25**, 925 (1969).
6 Burns, R. G., and Burns, V. M., *Handbook of Geochemistry* (edit. by Wedepohl, K. H.), (Springer, Heidelberg, Berlin and New York, 1970).
7 Gard, J. A., and Tait, J. M., *Acta crystallogr.*, **B28**, 825 (1972).
8 Smith, J. V., *Feldspar Minerals* (Springer, Heidelberg, Berlin and New York, 1974).
9 Rinaldi, R., Pluth, J. J., and Smith, J. V., *Acta crystallogr.*, **B31**, 1603 (1975).
10 Riley, P. E., and Seff, K., *Inorg. Chem.*, **13**, 1355 (1974).
11 Amaro, A. A., *et al.*, *Molecular Sieves* (edit. by Uytterhoeven, J. B.), 113 (Leuven University Press, Leuven, 1973).

Effect of glass dissolution on corrosion measurements

BOROSILICATE glass is widely used in electrochemical and corrosion studies because of its transparency, rigidity, ease of fabrication and, in particular, its resistance to chemical attack. Some disadvantages of the use of glass at very high temperatures, such as its solubility and the risk of solution contamination in autoclave studies of aqueous electrochemical processes, have been pointed out by Jones and Masterson[1]. In a previous communication[2] from this laboratory it was reported that at temperatures of 80 °C and above, appreciable dissolution of silica from glass can occur and that, in the corrosion of zinc, appreciable amounts of zinc pyrosilicate hydrate are present in the corrosion products. Later[3], figures for the silica contamination of distilled water held in glass vessels at 90 °C were quoted and the possible implication of glass dissolution on the corrosion behaviour of mild steel discussed.

Contamination of aqueous solutions by the test vessel is obviously undesirable in the acquiring of any standard corrosion data and this is particularly so in corrosion rate studies, since silicates—especially metasilicates—have an appreciable inhibitive action on metallic corrosion even when present in low concentrations. Since most of the experiments on the influence of temperature on corrosion rate—at least up to 100 °C and on occasions even above—have been conducted using glass test vessels considerable doubt must be placed on the validity of data obtained in this way.

Comparisons have been made of the corrosion behaviour of abraded mild steel sheet in distilled water at 90 °C using in the one case new 200 × 35 mm test tubes and condensers in borosilicate glass, as normally supplied commercially to laboratories, and in the other case equipment of similar geometry made in transparent fused quartz. Test specimens 30 × 25 × 0.8 mm were fully immersed in 60 cm³ of triply distilled water (pH 5.5, conductivity 84 µS m⁻¹) with the specimens resting on the bottom of the tubes at an angle of about 30° to the vertical. A slow stream of washed air was bubbled through the water just below its surface throughout the test. Specimens were tested in duplicate in separate vessels for periods of 28 and 100 d with the temperature maintained at 90 ± 1 °C throughout the test period.

Within the first few hours of immersion the specimens in glass were subjected to highly localised attack with hardly any rust in the water, whereas those in the quartz tubes had undergone more general corrosion, with the production of an appreciable amount of suspended rust. This difference in appearance of the specimens and the waters in the two types of vessel was maintained throughout the 100-d test period (Fig. 1). Measurements of dissolved monomeric silica, as determined by the colorimetric molybdate method, made on water after 8 d in similar tubes but without specimens present gave values of ~10 p.p.m. SiO_2 in water from glass but <2 p.p.m. SiO_2 in
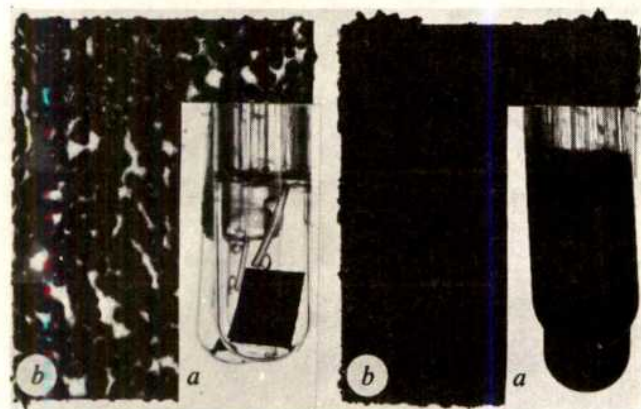
Fig. 1 *a*, Mild steel specimens in quartz and glass tubes after 28 d at 90 °C. Right, quartz tubes and "rusty" water; left, glass tubes and clear water. *b*, Specimens after removal from these tubes.

water from quartz vessels. Corrosion product was removed from specimens after test by cathodic treatment in 5 % H_2SO_4 + 0.1 % quinoline at 75 °C. Curves of weight loss as a function of time are shown in Fig. 2 and the appearance of typical specimens after removal from the test vessels but before removal of corrosion product are shown in Fig. 1.

We suggest that the presence of enhanced quantities of silica in the water in new glass vessels has resulted in a partial inhibitive effect on the corrosion process. The reason for the higher rate of silica dissolution from glass than from pure silica (quartz) is presumed to be the result of an initial dissolution of alkali metal ions from the glass producing local alkalinity which will facilitate subsequent dissolution of the Si–O network. The corrosion effects observed are, however, not thought to be associated with pH changes in the bulk of the water since the maximum pH values found in glass vessels—without specimens present—were only 7.1 after 28 d and 7.6 after 100 d.

These results have wide implications for corrosion tests in hot waters and will influence conclusions relating to the effect of temperature on the corrosion behaviour of steel, and pro-

Fig. 2 Corrosion–time curves for abraded mild steel in distilled water at 90°C. *a*, Specimens in quartz tubes; *b*, specimens in glass tubes.
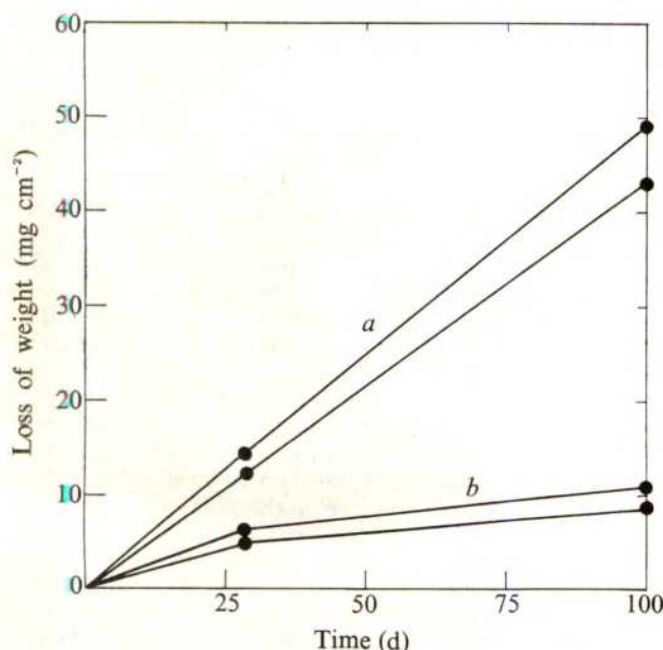
bably other metals, in these conditions. Because of the attenuation of corrosion rate with time shown in these experiments, the effect of glass dissolution may have a considerable bearing on the value, or even the existence, of a temperature for maximum corrosion below 100 °C.

Following the 28-d tests the glassware was cleaned in hot HCl, followed by thorough washing in water, and the tests repeated. The corrosion rates then measured over 28-d were similar to those obtained in quartz vessels, and furthermore, the amounts of silica dissolved from these used vessels were lower than those from new vessels. In view of the uncertainty of the effectiveness of any conditioning treatment, however, we consider it inadvisable to rely on this approach to avoid silica contamination of hot waters. We therefore recommend the avoidance of glass vessels for any accurate determinations of corrosion rates in such conditions.

Further work is in hand to determine the magnitude of the effects observed at temperatures below 90 °C.

G. BUTLER
A. D. MERCER

National Physical Laboratory,
Teddington, Middlesex, UK

[1] Jones, D. de G., and Masterson, H. G., in *Advance in Corrosion Science and Technology*, 1 (edit. by Staehle, R. W., and Fontana, M. G.), 1–49 (Plenum, New York and London, 1970).
[2] Butler, G., and Ison, H. C. K., *Nature*, **199**, 998 (1963).
[3] Brasher, D. M., and Mercer, A. D., *Br. Corrosion J.*, **3**, 120–129 (1968).

Unique form of tool-using in two gastropod molluscs (Trochidae)

TOOL-USING, the ability to manipulate an object as a functional extension of the body, has been described in a number of vertebrate and invertebrate species[1]. Although earlier workers regarded it as an indication of insight learning, its significance as a behavioural adaptation[2,3], possibly arising through the novel use of a pre-existing behaviour pattern[4], has been stressed in recent studies.

We report here a form of tool-using in two species of prosobranch gastropod, *Tegula brunnea* and *T. funebralis*, both of which inhabit the intertidal region on the Pacific coast of the US. This behaviour, unlike previously reported instances of tool-using, functions to alter the position of the organism itself rather than displace or modify an extraneous object, and to the best of our knowledge has never been observed in nature or reported from other laboratory studies.

Approximately 30 specimens of both species of *Tegula* (5.8–11.9 g) were collected by the Peninsula Marine Biological Company, Sand City, California, and maintained in our laboratory in a 30 gallon refrigerated Instant Ocean aquarium at 10 °C. They readily fed on brown kelp (*Egregia*).

When the snails were inverted, the propodium was extended over the edge of the shell and engaged in a brief period of probing. If they had access to a solid substrate or an object heavy enough to support their weight, the propodium adhered to the support and pulled the shell over in a manner similar to that reported for other gastropods[5,6]. A gravel substrate consisting of limestone pebbles having an average weight of 0.23 g and an average diameter of 6.0 mm could not sufficiently support the weight of an inverted snail. The prehensile anterior tip of the propodium would grasp a stone and lift it from the aquarium floor. Instead of releasing it immediately, however, the stone was conveyed in a posterior direction to the end of the foot through a series of alternate, retrograde undulations of the sole. This pattern is also typical of the normal locomotory movements of these species[7]. More stones were similarly procured and transferred to the posterior and median portion of the foot where they were accumulated (Fig. 1*a*). The continued
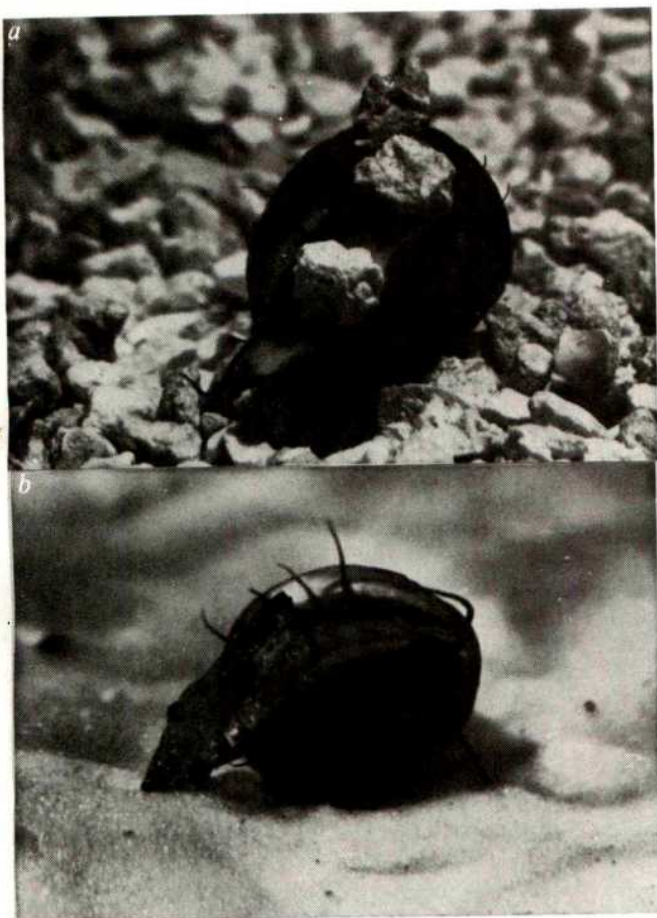
Fig. 1 *a*, Inverted specimen of *T. brunnea* in the process of transferring small stones to the posterior portion of the foot where they are accumulating. Note that the propodium is flexed downward and is ready to probe the substrate for another stone. The diameter of the widest whorl is approximately 2.5 cm. *b*, Inverted specimen of *T. brunnea* that is using a small flat stone as a shovel to gain the leverage to right itself on the sandy substrate.

upward flection movements of the propodium in acquiring stones and the rotating movements of the snail body with the additional weight afforded by the retained stones resulted in the gradual displacement and eventual righting of the snails. If larger stones approximately 35 % of the snail's weight were selected by the foot and extended to the side, the shell was induced to roll and the snail was promptly overturned. When an elongate stone was used, righting occurred by pushing it against the substrate in a shovelling fashion during the contortions and twisting movements of the snail body (Fig. 1*b*). Thus, these species of *Tegula* achieved the leverage necessary to bring the foot closer to the substrate by shifting their centre of gravity upward by the added weight of the retained stones and were able to restore themselves to an upright position.

The righting efficiency was affected and perhaps influenced by the nature of the supporting substrate, the position of the shell on the substrate, and the size of the stones used. There was little or no difference between the overall behavioural pattern of the two species, observed on approximately 60 occasions.

Both *T. brunnea* and *T. funebralis* possess a relatively heavy, turban-shaped shell which tends to overturn them after they have been dislodged from the substrate. The righting ability of these species of *Tegula* is significant as inverted snails unable to right themselves could not feed and would be particularly subject to predation by carnivorous echinoderms such as *Pisaster*, to which these species of *Tegula* respond by rapid flight movements[8].

T. funebralis, which has a patchy distribution on the Pacific coast of the US, is found in large numbers on moderately exposed rocky shorelines and is only encountered rarely in sandy areas[9]. At Mukkaw Bay, Washington, *T. funebralis* is particularly abundant in areas in which small cobbles and pebbles are present rather than solid bench rock[10,11]. From our observations of the Washington state coastline a few years ago, these small cobbles are several centimetres larger than the small stones used in our aquarium study. But as the snails move down the beach they may encounter substrata on which stone manipulation may have some adaptive significance. This would be particularly true in areas in which small stones or gravel would accumulate—for instance, on the bottoms of tidal pools and between larger rocks.

It has been suggested that the large operculum of strombid gastropods may have evolved partly to compensate for the lack of a large, long foot which could be used for righting movements[7]. We suggest that the stone manipulation as a form of tool-using may have similarly evolved as a compensatory behavioural adaptation.

PAUL J. WELDON
DANIEL L. HOFFMAN

Department of Biology,
Bucknell University,
Lewisburg, Pennsylvania 17837

1 Thorpe, W. H., *Learning and Instinct in Animals* (Methuen, London, 1963).
2 Goodall, J., *Nature*, **201**, 1264–1266 (1963).
3 Hall, K. R. L., *Curr. Anthrop.*, **4**, 479–494 (1963).
4 Alcock, J., *Evolution*, **26**, 464–473 (1972).
5 Weber, H., *Z. vergl. Physiol.*, **3**, 389–474 (1926).
6 Yonge, C. M., *J. mar. Biol. Assoc. UK*, **21**, 687–703 (1932).
7 Miller, S. L., *Proc. malac. Soc. Lond.*, **41**, 233–261 (1974).
8 Feder, H. M., *Ecology*, **40**, 505–512 (1963).
9 Stohler, R., *Veliger*, **6** (suppl.), 77–81 (1964).
10 Paine, R. T., *Ecology*, **50**, 950–961 (1969).
11 Paine, R. T., *Limn. Oceanogr.*, **16**, 89–98 (1971).

Significance of dorsoventral abdominal vibration among honey-bees (*Apis mellifera* L.)

IN observation hives worker honey-bees may often be seen to perform a behaviour pattern called dorsoventral abdominal vibration (DVAV)[1], the jerking dance[2], or shaking[3]. It has been suggested[4-6] that it serves to prepare bees for flight, mainly on the grounds that the only occasions on which queen honey-bees are vibrated (subjected to DVAV) are before their orientation and mating flights and before swarming. But conclusive evidence has not been provided, and von Frisch regards the function of DVAV as still being obscure[2]. Gahl[7] also leaves this question open.

The African bee, *Apis mellifera adansonii* L., is eminently suitable for study because, unlike European races, it has the habit of absconding if the nesting site becomes unfavourable for some reason. If DVAV does promote flight it should reach its highest level in a colony about to abscond, as this represents an extreme case of flight activity in which all the bees leave the nest together.

Detailed observations were made on two colonies in two-frame observation hives, one of which consisted of about 4,500 workers and the other of about 3,000. On the day of absconding the queens were not vibrated at all and the frequency of DVAV among the workers was extremely low. During numerous scans of the whole hive usually only one bee, and often none, could be seen performing DVAV at any time. DVAV is thus not a prerequisite for flight of either queens or workers.

This conclusion received support from observations made possible by another behavioural characteristic of the *adansonii* bee. In favourable temperature conditions foragers may be active at very low light intensities and the number leaving a hive often reaches the diurnal peak shortly after first light. In a colony of approximately 3,500 bees this exodus of foragers was compared on two occasions with the first DVAV performances
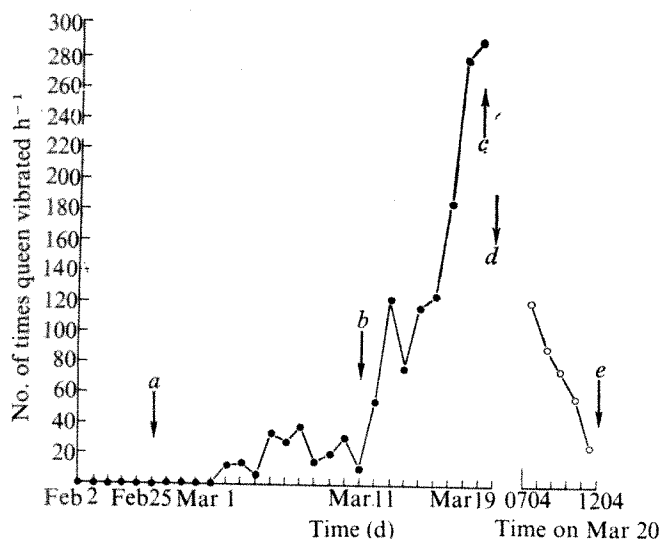
Fig. 1 Frequency with which a queen honey-bee was vibrated during a swarming cycle. *a*, First queen cup cell constructed. *b*, First egg laid in a cup cell. *c*, First queen cell sealed. *d*, Colony swarmed on March 20, 1975. *e*, Queen departed with swarm at 1204. The observation hive contained three Langstroth brood frames and the bees numbered 9,500–10,000 at the time of swarming. DVAV frequency was sampled for 90 min each day until the first queen cell was sealed. On the day of swarming recording was continuous.

of the day after the usual nocturnal cessation of the behaviour. In both cases well over 1,000 bees left the hive before the first DVAV was recorded. These observations do not agree with those of Allen[5] on European bees, in which it seemed that DVAV among workers probably preceded the first flights of the day.

As maximal flight activity was associated with minimal DVAV frequency in the above examples, it was postulated that DVAV is a flight inhibitor and not a flight activator as suggested by Hamman and Allen[4-6]. These mutually exclusive hypotheses were tested as follows.

Allen has shown that the frequency with which a queen is vibrated rises to a peak on the day of swarming[3]. It might reasonably be predicted from the activation hypothesis that this frequency would remain high right up to the time of the queen's departure with the swarm, whereas the inhibition hypothesis predicts that it would have to drop sharply before the queen could fly. Figure 1 shows that during the last 5 h before departure of a swarm the DVAV frequency did, in fact, decline rapidly. Compared with the peak of 291 vibrations h[-1] on the previous day there were only 25 during the last hour before swarming. Of these, only one occurred during the last 20 min. A similar result was obtained in a second trial and it agrees closely with a previously uninterpreted observation[1] that vibrations of the queen ceased altogether 30 min before a colony swarmed.

More direct evidence in favour of the inhibition hypothesis was obtained during a study of the behaviour of a virgin queen reared by a colony of about 1,500 bees. From the time of her emergence until the destruction of the last rival queen cell more than 8 h later this queen was not seen to be vibrated at all and she moved rapidly over the combs during the whole of this period except when being fed. She was first vibrated less than half an hour after the last queen cell had been attacked when, during a 6-min period, several bees vibrated her a total of twelve times. After the first three she slowed down considerably and after the next nine she stopped altogether for the first time. Thereafter, her activity diminished markedly (Table 1).

After repeating these observations on a second virgin queen with essentially similar results, it was concluded that DVAV is not exclusively a flight inhibitor, but a non specific activity inhibitor. It seems, furthermore, that one of its functions is the regulation of a queen's responses to the presence of queen cells. This latter conclusion was borne out by events recorded in two colonies that changed the direction of their behaviour from swarming to absconding.

Both colonies occupied two-frame observation hives and consisted of an estimated 4,500–5,000 bees. In the first, one sealed and two unsealed queen cells were discovered on December 17, 1974, but the colony was prevented from swarming by 4 consecutive days of overcast weather with intermittent rain and on day 5 the queen cells were destroyed. Figure 2 shows the frequency with which the queen was vibrated both during this
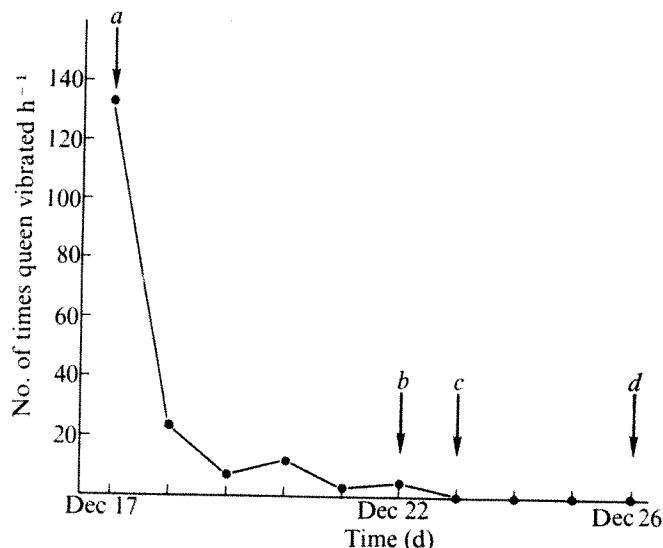

Fig. 2 Frequency with which a queen honey-bee was vibrated during a change in behaviour from swarming to absconding. *a*, Swarm cells discovered. *b*, Swarm cells destroyed. *c*, Colony attempted to abscond, but was prevented by a queen excluder at the hive entrance. *d*, Colony absconded after removal of the queen excluder.

period and on the following days until the colony absconded. The destruction of the cells coincided with the virtual cessation of DVAV, but it was not observed whether the queen initiated the attacks on them. In the second colony, however, the queen was seen to attack two of the three sealed queen cells present following a sharp drop in the frequency with which she was vibrated. In this case the cause of the change in behaviour was unknown, the weather being favourable to swarming at the time.

In view of these observations, it seems that the response of a mated queen to the presence of swarm cells is very similar to that of a virgin to rival queen cells. She is strongly motivated to destroy them, but is prevented from doing so by a reduction in the level of her activity through the agency of DVAV. The observed increase in the frequency with which she is vibrated as the swarming cycle progresses probably reflects her increasing tendency to attack the swarm cells as their occupants mature.

Table 1 Relationship between DVAV, presence of rival queen cells and movement of a virgin queen

Observation period		No. of occupied queen cells	No. of times queen vibrated	Movement of queen	
Starting time	Duration (min)			Duration (min)	%
0530	30	4	0	30	100
0824	30	3	0	30	100
1005	15	3	0	15	100
1100	10	3	0	10	100
1200	10	2	0	10	100
1307	30	2	0	30	100
1416	30	0	22	24	80
1540	10	0	4	7	70
1605	10	0	0	3	30
1705	10	0	0	2	20

Further evidence in favour of the inhibitory effects of DVAV on both queen and worker honey-bees, and the results of investigations into several of its functions will be reported in detail elsewhere.

D. J. C. FLETCHER

Department of Entomology,
University of Natal,
Pietermaritzburg, South Africa

1 Milum, V. G., *Am. Bee J.*, **95**, 97–104 (1955).
2 Frisch, K. von, *The Dance Language and Orientation of Bees* (Belknap, Cambridge, Massachusetts, 1967).
3 Allen, M. D., *Nature*, **181**, 68 (1958).
4 Allen, M. D., *Anim. Behav.*, **7**, 66–69 (1959a)
5 Allen, M. D., *Anim. Behav.*, **7**, 233–240 (1959b)
6 Hamman, E., *Insectes Sociaux*, **4**, 91–105 (1957).
7 Gahl, R. A., *Anim. Behav.*, **23**, 230–232 (1975).

Occurrence of "pockets" of very small cells in adipose tissue of the guinea pig

THE concept of hyperplastic obesity, wherein the excessive number of cells in the adipose depots is thought to originate during overnutrition in early life, has evolved in the laboratories of Hirsch[1], Salans[2] and Björntorp[3]. The estimation of the number of adipose cells relies on (1) a measurement of total body fat and (2) a measure of the mean cell size. The two methods most commonly used for measurement of cell size are based on the microscopic examination of cells in a piece of fixed tissue[4], or on sorting of cells in to size ranges by an electronic cell counter after dispersion of the tissue into a free cell suspension[5].

An advantage of the microscopic method is that the disposition of cells within the tissue can be observed directly. In general it seems that cells of different sizes, whether they follow a normal or a polymodal distribution, are randomly distributed throughout the tissue. Nevertheless our studies have revealed a distinctly heterogeneous distribution of fat cells in different adipose tissues of the guinea pig. We shall describe the occurrence of isolated "pockets" or clusters of cells very much smaller than the general size range within the tissue.

In these studies the guinea pigs were of the Frant strain, 3 months old, fed *ad libitum* Oxoid modified diet 18, with cabbage twice a week.

For cell sizing, a modification of the microscopic method of Sjöström *et al.*[4] was used. From each tissue site, four frozen sections (100 μm) were cut and in each section 10 cell diameters were measured in one direction and then 10 in another direction perpendicular to the first. Each measurement was the maximum diameter obtainable by focusing through the section. The volume of each cell was calculated by computer making the assumption that adipocytes are spherical[4,6]. No assumptions have been made about the distribution of the diameters, but the volume of each cell has been individually calculated and an average obtained from all the cells observed.

The volume of the adipocyte is essentially the volume of the triglyceride contained in it[4,5]. The proportion of tissue occupied by adipocytes is represented by its triglyceride content, determined gravimetrically after ether extraction.

We have defined a pocket as a cluster of four or more cells distinctly smaller than the general size distribution within the tissue (Fig. 1a and b). The clusters, which are sometimes seen around blood vessels, have been observed in all the tissues examined (three subcutaneous sites: interscapular, anterior and posterior abdominal, and three deep sites: parametrial or epididymal fat pads and perirenal adipose tissue). The following discussion relates only to the frequency of pockets in fat taken from the posterior abdominal region of a female guinea pig. No attempt has been
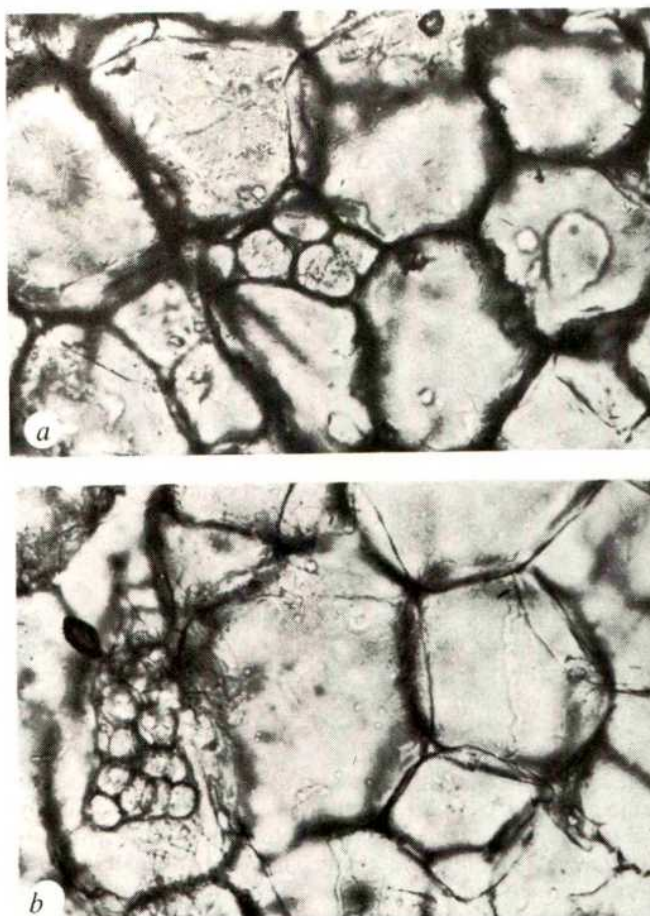
Fig. 1 *a*, Small pocket and surrounding "normal" cells. *b*, Large pocket and surrounding "normal" cells.

made to quantify pocket frequency at other sites although qualitatively it seems to vary from site to site.

Pocket frequency was determined by examining 10 serial sections of 100 μm thickness in a block of tissue measuring 6 × 11 × 1 mm (66 μl). All cells in a section were scanned and all pockets noted. In this particular block there were 90 pockets containing an average of 5.67 cells per pocket (maximum 9, minimum 4). Therefore there were 510 pocket cells in the tissue block.

The mean cell diameter of the "pocket cells" was 59 ± 11 (s.d.) μm (Fig. 2a) and of the "normal cells" 173 ± 31 (s.d.) μm (Fig. 2b). These data correspond to a cell volume of 0.11 nl (0.10 μg triglyceride per cell) for "pocket cells" and 2.72 nl (2.48 μg triglyceride per cell) for "normal cells". There is no overlap in the size ranges of the two population samples.

The triglyceride content of the tissue was 91 ± 3.1 % (± s.d. on six determinations). The volume of the tissue block occupied by fat cells was therefore 60.060 μl.

The number of cells in the 66-μl block of tissue is essentially the volume occupied by cells divided by the mean volume per cell. The presence of pocket cells complicates the calculation because of the difficulty of determining mean cell volume. The calculation has been done three ways.

(1) The volume occupied by "normal" cells is obtained by subtracting the volume occupied by 510 "pocket" cells from the total volume of tissue occupied by fat cells. The number of "normal" cells in this space is therefore 22,093 and the total number of cells is 22,603.

(2) If the observer recognises the existence of pocket cells but chooses to ignore them, the total cell number would be underestimated by 490 or 2.2 %.
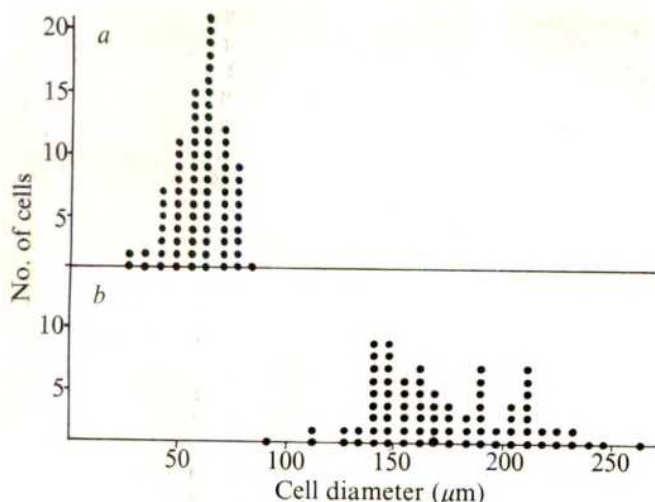
Fig. 2 *a*, Size distribution of "pocket" cells. *b* Size distribution of "normal" cells.

[4] Sjöström, L., Björntorp, P., and Vrána, J., *J. Lipid Res.*, **12**, 521–530 (1971).
[5] Hirsch, J., and Gallian, E., *J. Lipid Res.*, **9**, 110–119 (1968).
[6] Lemmonier, D., *J. clin. Invest.*, **51**, 2907–2915 (1972).
[7] Ashwell, M. A., Priest, P., and Sowter, C., *Nature*, **256**, 724–725 (1975).

Importance of fixed sections in the study of adipose tissue cellularity

IN the study of obesity in human subjects and animal models attention has recently been directed to the size and number of cells in adipose tissue. Measurements of fat cell sizes have led to the classification of hypertrophic, hyperplastic or mixed forms of obesity in animals[1] and man[2]. A study of the literature concerning animals, however, reveals wide discrepancies in reports of the degree of hypertrophy or hyperplasia contributing to the expansion of fat mass observed in genetically[3,4] or experimentally obese animals[1,5–10]. These discrepancies have arisen not only because different species and strains of animals were used and different fat depots were sampled, but also because different methods were used to determine fat cell size and number.

Two of the most popular methods used for determining fat cell size are the osmium fixation of fat cells[11] and the sizing of isolated cells prepared by collagenase digestion[12,13]. Both methods can be used to determine the distribution of cell sizes within a population and will yield a value for the mean cell diameter of the population. Neither method, however, gives any information about the *in situ* distribution of cell sizes or the
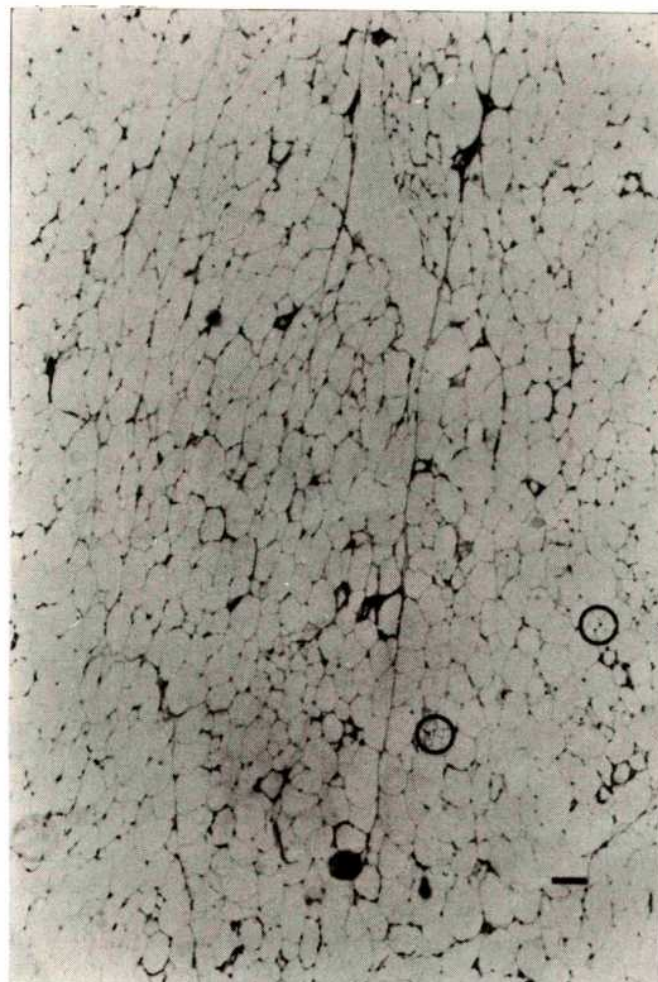
(3) If the observer includes a pocket of, say, four cells in his measurements, the calculated mean cell diameter would thereby be reduced and the total number of cells would be overestimated by 622 or 2.8%.

The results of this study reveal a distinct population of small adipocytes in the adipose depots of the guinea pig. Their mean volume per cell is only 4% that of "normal" cells and they represent only 2.3% of the cell population in terms of numbers and constitute only 0.09% of the volume occupied by adipocytes. The error introduced therefore, by inclusion or omission of these cells during the microscopic determination of cell size is small. The paper by Ashwell *et al.* which follows[7] indicates, however, that the pocket phenomenon is more widespread than hitherto realised and we cannot rule out the possibility that in some tissues the frequency may be much higher rendering the microscopic method of determining mean cell size of dubious value.

But the results do highlight the desirability of microscopic examination of adipose tissue. Techniques based on sorting cells after digestion of the tissue would certainly reveal polymodality but would not distinguish between random occurrence of small cells throughout the tissue and the clustering of small cells in pockets as observed here. Moreover by detailed microscopy it may be possible to determine whether the pockets themselves are randomly distributed in the tissue, or whether their association with blood vessels is more than fortuitous. This raises the question of interpretation of the pockets in terms of tissue development. Preliminary observations suggest to us that pocket frequency decreases with age and this may indicate the pockets represent loci of most recent fat cell recruitment from a bed of pre-adipocytes. This possibility is being investigated by autoradiographic examination of tissue exposed to tritiated thymidine.

JANET KIRTLAND
MICHAEL I. GURR
GEOFFREY SAVILLE

*Division of Nutrition and Agriculture,
Unilever Research Laboratory,
Colworth House, Sharnbrook, Bedford MK44 1LQ, UK*

ELSIE M. WIDDOWSON

*Department of Investigative Medicine,
Cambridge CB2 1QN, UK*

[1] Hirsch, J., and Knittle, J. L., *Fedn Proc.*, **29**, 1516–1521 (1970).
[2] Salans, L. B., Cushman, S. W., and Weismann, R. E., *J. clin. Invest.*, **52**, 929–941 (1973).
[3] Björntorp, P., and Sjöström, L., *Metabolism*, **20**, 703–713 (1971).

Fig. 1 Subcutaneous adipose tissue from a 4-month-old obese mouse. Fixed in Bouin's fixative and thin sections stained with haematoxylin and eosin. Scale marker, 100 μm.
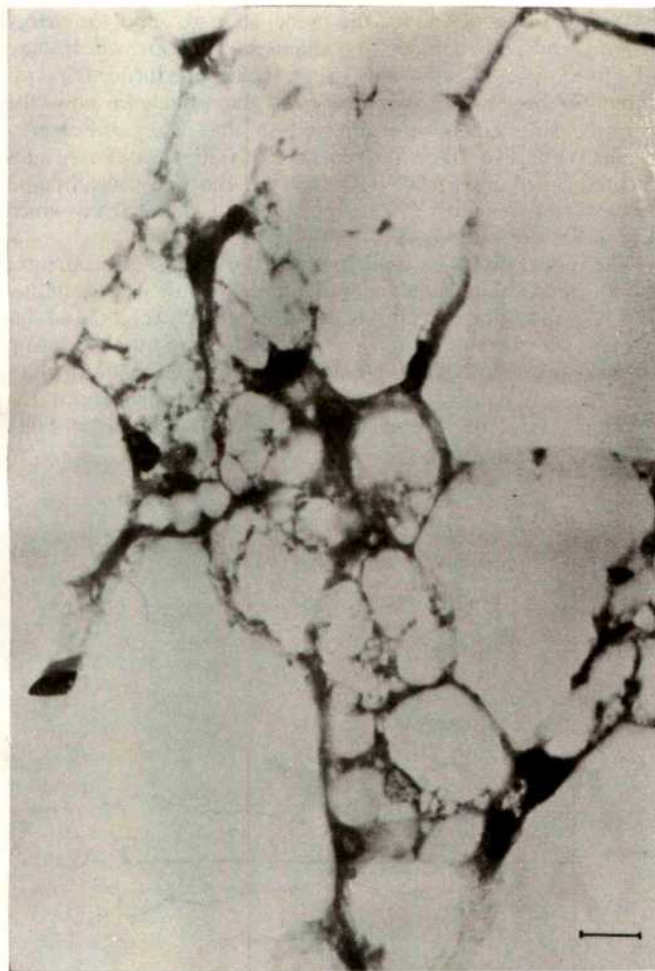
Fig. 2 Details as for Fig. 1. Scale marker, 10 μm.

histological or pathological changes that might occur in the adipose tissue. We have studied numerous samples of adipose tissue from various sites of genetically obese mice and their lean littermates, and we propose that examination of fixed sections is essential if maximum information about adipose tissue cellularity is required.

Figure 1 shows a low power photograph of a fixed section of subcutaneous adipose tissue from a 4-month-old genetically obese mouse (C57BL.6J.ob/ob). The black circles show "pockets" of small cells which have developed between areas of larger cells. Figure 2 shows a high power photograph of one pocket. These pockets are particularly evident in the perirenal and subcutaneous fat depots and are not quite so obvious in the epididymal or parametrial depots. We have examined fat from obese mice from 4 to 40 weeks old and have seen pockets at all ages in obese mice.

A photograph with a final magnification of ×210 showing the same field as Fig. 1 was used for sizing the fat cells. A representative area of large cells and pocket cells was chosen and this contained 153 cells. Cell diameters were measured with a ruler in two directions for each cell: the maximum diameter and the diameter perpendicular to this. The average of these two diameters was calculated and three determinations of mean cell diameter ±s.e.m. were made. For all cells, mean cell diameter was 70.5 μm ± 2.96; for normal cells, mean cell diameter was 89.2 μm ± 2.66; for pocket cells, mean cell diameter was 28.3 μm ± 0.72.

Several studies of adipose tissue development have concentrated on development of the gonadal fat pads (for example, ref. 14) probably because these are a very convenient source of tissue. Our observations on obese mice and also on normal rats have convinced us that the gonadal fat pads are unrepresentative

of other fat depots in animals and are certainly unrepresentative of human adipose tissue. Conclusions drawn from their study should be interpreted with caution. The photographs of fixed sections leave no doubt that pockets of small cells exist in some fat depots and it is tempting to speculate that these pockets represent loci for new fat cell formation. The development of adipose tissue has usually been studied in young growing animals and so far there is no general agreement as to the origin of fat cell precursors. Some investigators believe that fat cells arise from ordinary fibroblasts and others believe that they arise from special connective tissue cells, distinct from fibroblasts (see review in ref.15). A detailed study of the location of these pockets of small cells should yield more information in this field.

We thank Miss Janet McKenzie for the photomicrographs and Dr M. Cawthorne, Beecham Research Laboratories for obese mice.

MARGARET A. ASHWELL
PAULINE PRIEST
CHRISTOPHER SOWTER

Clinical Research Centre,
Watford Road,
Harrow, Middlesex HA1 3UJ, UK

1 Johnson, P. R., and Hirsch, J., *J. Lipid Res.*, **13**, 2–11 (1972).
2 Salans, L. B., Cushman, S. W., and Weismann, R. E., *J. clin. Invest.*, **52**, 929–941 (1973).
3 Lemonnier, D., *J. clin. Invest.*, **51**, 2907–2915 (1972).
4 Herberg, L., Döppen, W., Major, E., Gries, F. A., *J. Lipid Res.*, **15**, 580–585 (1974).
5 Hellman, B., Taljedal, I. B., and Westman, S., *Acta Morphol. scand.*, **5**, 182–189 (1962).
6 Lemonnier, D., Winand, J., Furnelle, J., and Christophe, J., *Diabetologia*, **7**, 328–333 (1971).
7 Hausberger, F. X., in *Handbook of Physiology. Adipose Tissue*, 519–528 (Am. Physiol. Soc., Washington, DC, 1965).
8 Bray, G. A., *Proc. Soc. exp. Biol. Med.*, **131**, 1111–1114 (1969).
9 Rakow, L., Beneke, G., Mohr, W., and Brauchle, I., *Beitr. Path.*, **143**, 301–311 (1971).
10 Johnson, P. R., Zucker, L. M., Cruce, J. A. F., and Hirsch, J., *J. Lipid Res.*, **12**, 706–714 (1971).
11 Hirsch, J., and Gallian, E., *J. Lipid Res.*, **9**, 110–119 (1968).
12 Rodbell, M., *J. biol. Chem.*, **239**, 375–380 (1964).
13 Di Girolamo, M., and Mendlinger, S., *Am. J. Physiol.*, **221**, 859–864 (1971).
14 Greenwood, M. R. C., and Hirsch, J., *J. Lipid Res.*, **15**, 474–483 (1974).
15 Barrnett, R. J., in *Adipose Tissue as an Organ* (edit. by Kinsell, L. W.), 3–78 (Thomas, Illinois, 1962).

Pattern of fat and lean tissue deposition in children

THERE is evidence[1] of a prolonged change in body composition in children who have been overfed during the first year of life, and it has been suggested[1,2] that there may be a critical period for fat deposition and adipocyte formation. We have examined published data on growth and body composition of normal children, and have found that these reveal cyclical changes in fat and lean tissue deposition which confirm the existence of critical periods, and offer an explanation for the observed changes in body composition.

From tables of average body weight extending from 13 weeks after conception[3] and from birth to 17 yr (ref. 4), we have calculated increments in body weight per unit time (ΔM). Similarly, the increments in body fat mass (ΔF) can be calculated from data on average body composition at various ages[3,5]. The difference between ΔM and ΔF at any given age is taken to be the change in lean tissue (ΔL). Figure 1 shows how the proportion of lean tissue deposited ($\Delta L/\Delta M$) varies with age throughout the growth period.

The changes shown are large and cyclical, and are unlikely to be artefacts in spite of the use of data from different sources for the pre- and postnatal periods. In early and midfoetal life, the deposited tissue is almost entirely lean, but fat deposition is favoured nearer full term. Throughout the first year of life, fat deposition predominates, but the proportion of lean rises to a maximum again at about 4 yr, when very little of the weight gained is deposited as fat. This cycle is repeated twice more,
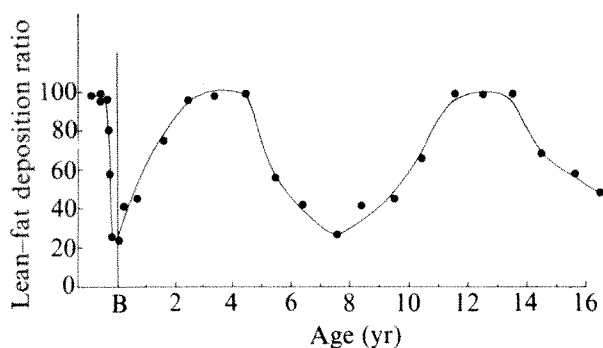
Fig. 1 Variations with age in the ratio between lean (ΔL) and fat (ΔF) deposition in normal males aged from 26 weeks after gestation to 16 yr.

We have now developed the model so as to simulate energy balance and body composition changes during growth. Instead of a fixed ratio of lean to fat tissues, the time sequence of ratios shown in Fig. 1 is programmed into the model. Initially, the various parameters were adjusted so that when the energy intake is made to follow the sequence of values at different ages recommended by FAO/WHO (ref. 6), the body weight and composition remain close to the standard values from which Fig. 1 was derived.

The model was then used to study the effects of departures from the recommended energy intake. Thus, for example, Fig. 2 shows the deviations from expected weight and fat content which result from over- or underfeeding by 20% during the first year after birth. Note that the effects on body composition are marked, and that they persist throughout the following 9 yr of 'normal' feeding even though total body

with a return to predominantly fat deposition during the period between 6 and 10 yr, lean again between 12 and 14 yr (the period of the adolescent growth spurt), finally swinging back to fat deposition at 17 yr. In adults, changes in body composition consequent on experimental underfeeding, indicate that the lean–fat deposition ratio varies from about 50% for 'lean' types of individual down to 5% for 'obese' types.

In a computer model, which simulates weight maintenance in the adult, food intake energy is compared each day with energy expended for maintaining metabolic activity and for physical work (P.R.P. and A.E.D., unpublished). When positive, the difference is deposited as fat and lean tissue in proportions which are fixed for a particular physiological type of person (that is, tending to be 'lean' or 'obese'). If the daily energy balance is negative, sufficient tissues are mobilised to make up the deficit, but still in the same proportions. The model uses a random number generator to simulate the effects of daily variations in intake and expenditure, and predicts the time course of changes in body weight and composition that will result from feeding specified levels of daily energy intake.

Fig. 3 Predicted deviations from expected mass for age when ratio of lean–fat tissue deposition is altered by $\pm 10\%$, and a normal calorie intake is given. $\triangle$ and $\bigcirc$, Fat and total body masses with a 10% reduction in the lean–fat ratio. $\blacktriangle$ and $\bullet$, Fat and total body masses with a 10% increase in lean–fat ratio.

weight rapidly returns to near the expected values. This confirms clinical findings by emphasising the importance of over- or underfeeding during early life.

The programmed sequence of lean–fat tissue deposition ratios can also be changed. Thus, Fig. 3 shows the effects of changes $\pm 10\%$ imposed on all the values shown in Fig. 1, while maintaining energy intakes at the normal recommended levels. The upward shift produces a lean, ectomorphic individual; the downward, a plump endomorph. As has been mentioned above, there is experimental evidence that leanness or obesity in adults is associated with different values of the ratio $\Delta L/\Delta M$, and these studies support the idea that changes in the 'settings' of the ratio may explain many of the observed features of the development of ecto- and endomorphy in children. The metabolic mechanisms and the degree of genetic dependence of the ratios are unknown, but will clearly be important areas for future research.
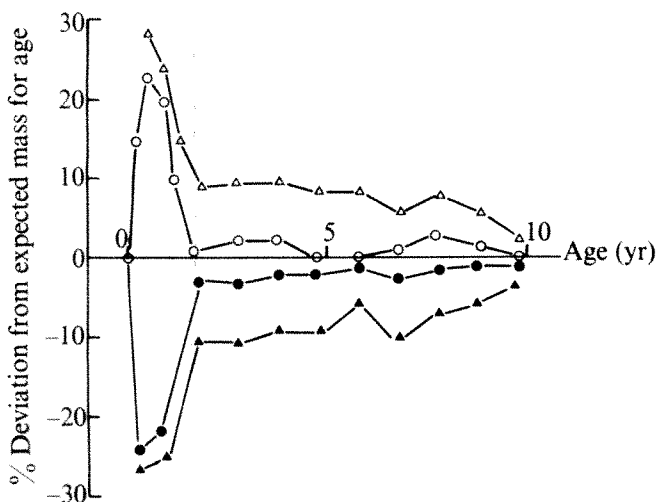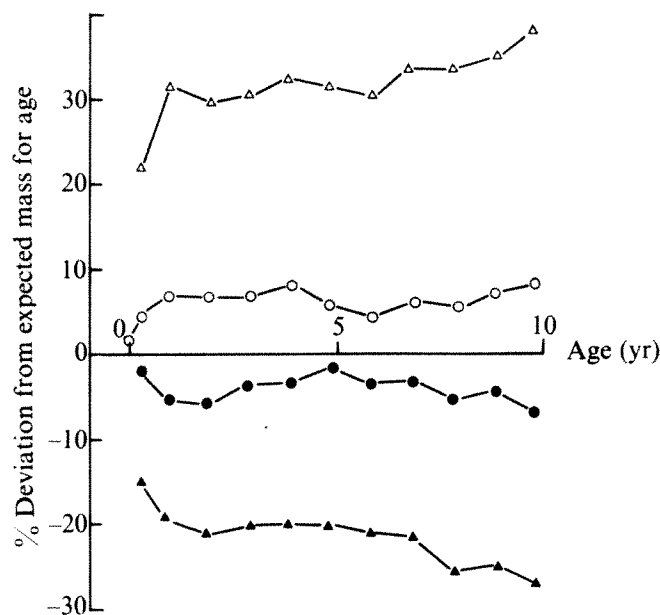
Fig. 2 Predicted changes in body mass in children who have been given a 20% deficit or excess in calories from birth to 1 yr and then a normal calorie intake. Changes are shown as percentage of expected mass for age. $\triangle$, and $\bigcirc$, Fat mass and total body mass with excess calories. $\blacktriangle$ and $\bullet$, Fat and total body mass with a calorie deficit.

A. E. DUGDALE

Department of Child Health,
University of Queensland,
St Lucia, Queensland, Australia

P. R. PAYNE

Department of Human Nutrition,
London School of Hygiene and Tropical Medicine,
London WC1, UK

1 Brook, C. G. D., Lloyd, J. K., and Wolf, O. H., *Br. med. J.*, ii, 25 (1972).
2 Boulton, T. J. C., Dunlop, M., and Count, J. M., *Aust. Paed. J.*, 10, 301 (1974).
3 Widdowson, E., in *Scientific Basis of Paediatrics* (Heinemann, London, 1974).
4 *Documenta Geigy Scientific Tables*, seventh ed. (Geigy, Basle, 1970).
5 Rauh, J. L., and Schumsky, D. A., in *Human Growth* (Lea and Febiger, Philadelphia, 1968).
6 *Energy and Protein Requirements* (Report FAO/WHO Ad Hoc Expert Committee Wld Hlth Org. tech. Rep. Ser., no. 522, 1973).

Eosinophils as mediators of antibody-dependent damage to schistosomula

WE have reported previously[1] that damage to schistosomula can be assayed by measuring release of ^{51}Cr from labelled larvae. Using this technique, we have shown that schistosomula can be damaged by a combination of normal human peripheral blood leukocytes and of heat-inactivated sera from patients infected with *Schistosoma mansoni*[1]. We now present evidence that the eosinophil is substantially the most active mediator of damage in normal peripheral blood, but that cells from eosinophilic patients are relatively inactive.

Schistosomula were prepared by allowing *S. mansoni* cercariae to penetrate mouse skin *in vitro*[2], and were stored overnight at 4 °C before labelling with ^{51}Cr as previously described[1]. Sera from five patients infected with *S. mansoni* were used as sources of anti-schistosomular antibody in different experiments. These sera had previously been tested for cell-dependent cytotoxic activity to schistosomula, and dilutions (1/24 to 1/90) were chosen which had given at least 50% cytotoxicity in the presence of unpurified leukocytes at an effector to target ratio of 2,000 : 1 over an 18-h incubation period. All sera were inactivated at 56 °C for 1 h before use, and were diluted in HEPES-buffered Eagle's minimal essential medium containing 10% heat-inactivated

foetal calf serum (MEM/FCS). Effector cells from uninfected laboratory workers were prepared from heparinised blood by sedimentation of 5 volumes of blood with 1 volume of 4.5% dextran in phosphate-buffered saline for 30 min at 37 °C. Cells from the leukocyte-rich supernatant were washed twice in MEM/FCS, and were subjected to various purification procedures before testing for cytotoxicity. Eosinophil counts in the peripheral blood of the four normal subjects studied ranged from 90 to 240 mm^{-3}. Unpurified leukocyte-rich preparations after dextran sedimentation contained 3–6% eosinophils. The cytotoxicity test was carried out in 7×38 mm sterile plastic tubes : each of four replicate tubes contained 0.1 ml of labelled schistosomula (500 ml^{-1}), 0.1 ml of diluted serum, and 0.1 ml of effector cells at various concentrations. After overnight incubation, half of the supernatant was withdrawn, and percentage isotope release was calculated from the count rates in both the pellet and the supernatant tubes. Statistical analyses were carried out on the logarithmically-transformed percentage isotope release data, using multifactorial analysis of variance and Duncan's multiple range tests[3]. Each of the experiments described here was repeated at least three times, with comparable results.

Titration of unpurified peripheral blood leukocytes for their ability to induce release of ^{51}Cr from schistosomula in the presence of antibody revealed that isotope release was proportional to the logarithm of the effector to target ratio over a range of ratios from 50 : 1 to 5,000 : 1 (Fig. 1). The high ratios required presumably reflect the multicellular nature of the target organism.

Subsequent attempts to purify the effector cell showed that cytotoxic activity was progressively lost during repeated cycles of incubation with carbonyl iron and magnetic extraction. Although recent work[4] has shown that some antibody-dependent effector cell activities in the mouse may be removed by incubation with uncoated but not with protein-coated carbonyl iron particles, this finding was initially taken to imply that the effector cell was phagocytic. It was then found that after centrifugation of the starting effector cell preparation over Ficoll–Hypaque[5], the cells recovered from the pellet were approximately 10 times more active than those recovered from the interface (Fig. 2). For example, pellet cells induced 33% isotope release at a cell to target ratio of 90 : 1, whereas for the interface cells a ratio of 1,020 : 1 was required to induce the same level of release. The differential counts for the two cell preparations (legend to Fig. 2) show that the pellet preparation contained 88% polymorphonuclear cells (PMN) and 12% mononuclear cells (MN), whereas the interface contained 9% PMN and 91% MN. From these differential counts, it can be calculated that the ratio of PMN to targets in the preparation of pellet cells which gave 33% release

Table 1 Reduction of cytotoxicity after pretreatment of effector cells with ANS or AES

Treatment	Complement	ANS + complement	AES + complement
Ratio of total effector cells to targets required for 30.5% isotope release	530	1,000	3,200
% reduction in cytotoxicity per added cell	0	47	83.5
% eosinophils killed	5	20	80
% neutrophils killed	5	100	10
*Ratio of viable eosinophils to targets in preparations giving 30.5% release	20	32	26
*Ratio of viable neutrophils to targets in preparations giving 30.5% release	302	0	1,728

*Based on a differential count in the complement control of 4% eosinophils, 60% neutrophils, and 36% mononuclear cells.

Fig. 1 Titration of effector activity of unpurified peripheral blood leukocytes, containing 62% neutrophils, 5% eosinophils and 33% mononuclear cells. Abscissa: $\log_{10}$ ratio of unpurified effector cells to target schistosomula. Ordinate: percentage isotope release from schistosomula after 15 h incubation. Analysis of variance: "dilution" effect $P < 0.001$ ($F = 16.7:9$ and 30 degrees of freedom). Multiple range tests:"antibody alone" significantly less ($P < 0.05$) than 200:1 ratio and all higher ratios.
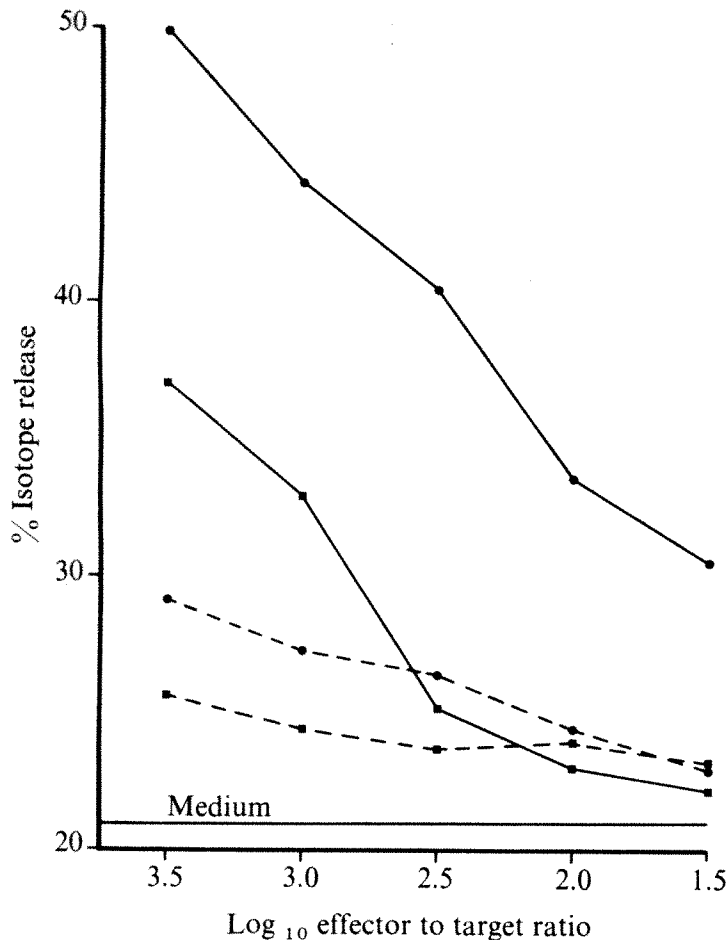
Fig. 2 Titration of effector activity of partially purified polymorphonuclear leukocytes and mononuclear cells. Cells from the leukocyte-rich supernatant of a heparinised, dextran-sedimented peripheral blood preparation were washed twice in MEM/FCS and overlayed on 10 ml of Ficoll-Hypaque[b]. This preparation was centrifuged at 400g for 40 min, and the interface layer and the pellet were washed twice in MEM/FCS before dilution and testing for cytotoxicity in the presence (————) or absence (— — —) of antibody. ●, Pellet cells, containing 8% eosinophils, 80% neutrophils and 12% mononuclear cells; ■, interface cells, containing 0.7% eosinophils, 8% neutrophils and 91% mononuclear cells. Analysis of variance: ("with antibody" only): "purification" effect $P < 0.001$ ($F = 100.6:1$ and 30 degrees of freedom). Interaction "purification" × "cell dilution" $P > 0.05$ ($F = 0.92:4$ and 30 degrees of freedom), indicating that the slopes of the titration curves are not significantly different.

was almost identical to the PMN to target ratio in the interface preparation which gave the same level of release (79 : 1 and 89 : 1 for the pellet and interface preparations respectively). In contrast, the ratios of MN to targets in these two preparations differed by two orders of magnitude (11 : 1 and 928 : 1 for the pellet and interface preparations respectively). Therefore, for a mononuclear cell to be responsible for damage, there would have to be a 100-fold selective purification of that particular mononuclear cell into the pellet. No evidence for this was available from other systems: it was therefore concluded that the active cell was a member of the PMN series. Since, however, both neutrophils and eosinophils were enriched to a comparable extent in the pellet (legend to Fig. 2), it was not possible at this stage to conclude which type of PMN was active.

The anti-human eosinophil serum (AES) and anti-human neutrophil serum (ANS)[6,7] were then used in an attempt to identify the particular PMN involved. Unpurified affector cells were treated with AES or ANS and complement, and after subsequent washing were titrated for antibody-dependent cytotoxicity to schistosomula (Fig. 3 and Table

1). The AES and ANS reagents were also tested for direct cytotoxicity to purified neutrophils[7] and eosinophils[8] by Trypan blue exclusion (Table 1). The number of total effector cells in each preparation required to induce equivalent levels of isotope release was calculated from Fig. 3. Since the release induced by the AES-treated preparation was so low, the level chosen for comparison (30.5%) was that induced by the AES-treated sample at the highest cell concentration tested. From the total number of cells required, the reduction in cytotoxic activity per added cell could then be calculated for each preparation (Table 1). This reduction was related to the reduction in viable eosinophils, but not to the reduction in viable neutrophils. Equivalent levels of isotope release were induced by preparations containing comparable numbers of viable eosinophils, whereas there was no relationship between isotope release and the number of viable neutrophils (bottom two lines of Table 1). Since the starting preparation contained 4% eosinophils and 60% neutrophils, it could be calculated that each eosinophil was at least 27 times more active than each neutrophil in mediating cytotoxicity. Furthermore, the sum of the inhibitions induced by AES and ANS alone was greater than 100%, and part of the ANS effect may be artificial, since it may have been attributable to competitive inhibition of eosinophil cytotoxicity against antibody-coated schistosomula by the large numbers
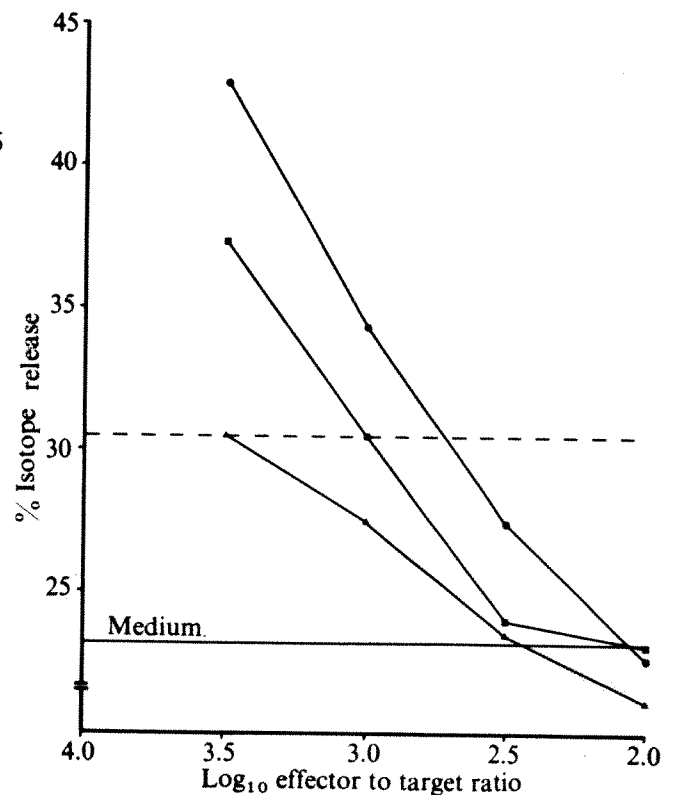

Fig. 3 Reduction of cytotoxicity after pretreatment of effector cells with AES and ANS. Unpurified leukocytes from a heparinised, dextran-sedimented peripheral blood preparation were washed twice in MEM/FCS. Aliquots were treated with MEM/FCS alone (●), with AES absorbed once with neutrophils and four times with erythrocytes, at a 1/10 final dilution (▲), or with ANS absorbed four times with erythrocytes, at a 1/10 final dilution (■). All preparations were incubated for 30 min and washed once with MEM/FCS. Fresh guinea pig serum (1/6 final dilution) was then added, and the preparations were incubated for a further 45 min at 37 °C before washing and testing for cytotoxicity to antibody-treated schistosomula. Analysis of variance: "antiserum effect" $P < 0.001$ ($F = 22.2:2$ and 36 degrees of freedom). Interaction "antiserum" × "cell dilution" $P > 0.05$ ($F = 2.35:6$ and 36 degrees of freedom), indicating that the slopes of the titration curves are not significantly different.

of antibody-coated neutrophils present in the preparation. This argument does not apply to the AES effect, since the numbers of antibody-coated eosinophils present in the AES-treated preparation would be too small to inhibit the much larger numbers of neutrophils. The inhibition induced by ANS therefore was more likely to be artificial than that induced by AES.

In contrast to these results, cells from three patients with marked eosinophilia (21% to 38%) induced by schistosomiasis or other helminth infections did not show the increase in cytotoxic activity which would be predicted if all eosinophils were equally active. One interpretation is that eosinophils from such patients may be blocked or inactivated by the immune complexes that elicited the eosinophilia. This possibility is now being tested.

These findings indicate that the major, and possibly the only, cell type in normal human peripheral blood capable of inducing antibody-dependent, complement-independent damage to schistosomula is the eosinophil. Other mechanisms for damaging schistosomula in vitro have been described[2,6-11], but the eosinophil-mediated reaction described here is potentially of particular importance in the light of subsequent findings (A.A.F.M., K. S. Warren and P. A. Peters, unpublished) that administration of AES abolishes resistance to reinfection in mice infected with S. mansoni. The observations described here, therefore, not only reflect a new eosinophil activity, but may also provide a useful model for analysing one mechanism of immune damage to schistosomula that does have relevance in vivo.

This work was supported by the Wellcome Trust, and formed part of a collaborative project with the World Health Organization.

A. E. BUTTERWORTH
R. F. STURROCK
V. HOUBA

Wellcome Trust Research Laboratories,
PO Box 43640, Nairobi, Kenya

A. A. F. MAHMOUD

Case Western Reserve University School of Medicine,
Cleveland, Ohio

A. SHER

Peter Bent Brigham Hospital,
Boston, Massachusetts

P. H. REES

Department of Medicine,
University of Nairobi, Kenya

1 Butterworth, A. E., Sturrock, R. F., Houba, V., and Rees, P. H., Nature, 252, 503 (1974).
2 Clegg, J. A., and Smithers, S. R., Int. J. Parasit., 2, 79 (1972).
3 Butterworth, A. E., Cell. Immun., 16 (1975).
4 Sanderson, C. J., Clark, I. A., and Taylor, G. A., Nature, 253, 376 (1975).
5 Boyum, A., Scand. J. clin. Lab. Invest., 21, suppl 97, 77 (1968).
6 Mahmoud, A. A. F., Kellermeyer, R. W., and Warren, K. S., New Engl. J. Med., 290, 417 (1974).
7 Mahmoud, A. A. F., Kellermeyer, R. W., and Warren, K. S., Lancet, ii, 1163 (1974).
8 Day, R. P., Immunology, 18, 955 (1970).
9 Dean, D. A., Wistar, R., and Murrell, K. D., Am. J. trop. Med. Hyg., 23, 420 (1974).
10 Perez, H., thesis, Brunel University (1974).
11 Capron, A., Dessaint, J. P., Capron, M., and Bazin, H., Nature, 253, 474 (1975).

Mediation of serum-induced changes in cyclic nucleotide levels in cultured fibroblasts by cyclic nucleotide phosphodiesterase

CELL cultures of untransformed fibroblasts can be maintained in a stationary or relatively quiescent state by adjusting serum concentrations to suboptimal levels[1]. Addition of excess serum to quiescent cell cultures causes a rapid decrease in the intracellular concentration of cyclic AMP and a rapid increase in cyclic GMP levels[2-5]. These alterations in cyclic nucleotide concentrations precede the
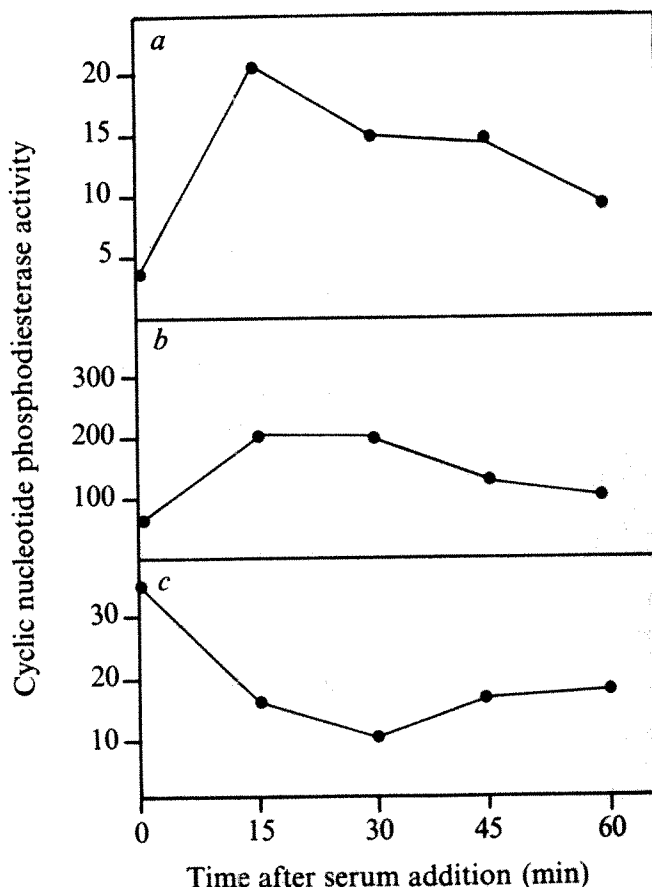

Fig. 1 BHK 21/c13 cells from surface cultures in 75 cm² Falcon tissue flasks were washed once with Eagle's minimal essential medium (MEM) containing 1% foetal calf serum. After trypsinisation (0.25% trypsin) cells suspensions were made in MEM and 1% foetal calf serum. 1 × 10⁶ cells were seeded in 75 cm² Falcon culture dishes with 10 ml of MEM and 1% foetal calf serum and were incubated for 48 h at 37 °C. All cell cultures were then supplemented with an additional 10% foetal calf serum and incubated for the indicated times after which they were collected by scraping and centrifugation at 500g for 10 min. The cells were washed twice in Hanks' balanced salt solution; homogenised in 40 mM Tris (pH 8.0), and cyclic AMP and cyclic GMP phosphodiesterase activities determined according to the methods of Thompson and Appleman[9,10]. Four culture dishes were pooled for each enzyme activity determination. The cyclic nucleotide activity is shown in pmol per mg protein per min. These data illustrate the results of a typical experiment. a, Cyclic AMP phosphodiesterase activity measured at a cyclic AMP concentration of 1.0 µM; b, cyclic AMP phosphodiesterase activity measured at a cyclic AMP concentration of 50 µM; c, cyclic GMP phosphodiesterase measured at a cyclic GMP concentration of 1.25 µM.

changes in macromolecular synthesis (such as RNA, protein and DNA synthesis) and growth induced by serum, and constitute one of the earliest steps in the sequence of events before the initiation of growth. The mechanism responsible for altering cyclic nucleotide concentrations in response to serum or other growth stimuli has not been elucidated however. We show here that a rapid alteration in cyclic nucleotide phosphodiesterase activity occurs when serum is added to quiescent fibroblasts and demonstrate that this alteration could mediate the changes in cyclic nucleotide levels.

Figure 1 illustrates the changes in phosphodiesterase activities seen after addition of 10% foetal calf serum to BHK 21/c13 (BHK cells, 1 × 10⁶ cells per dish) cultured in media containing 1% foetal calf serum. Fig. 1a shows a 4–5-fold increase in cyclic AMP phosphodiesterase activity, measured at 1 µM cyclic AMP concentrations, within the first 15 min of serum addition. The total cyclic GMP
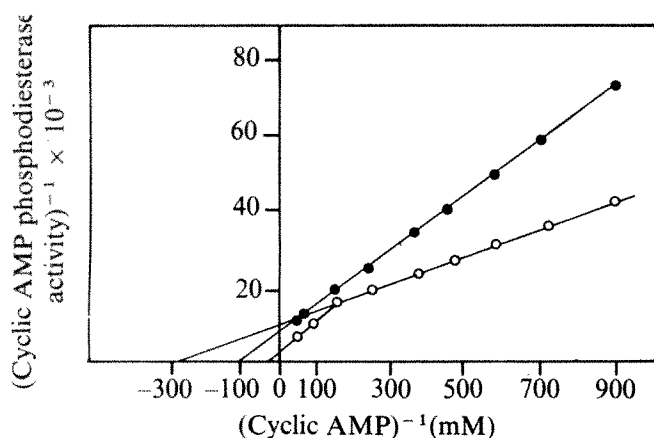
Fig. 2 Cells from ten culture dishes (1 × 10⁶ cells per 75 cm² grown and collected as described in Fig. 1) were pooled, enzyme activities determined, and Lineweaver–Burk plots analysed. ●, Cells in 1% serum for 36 h; ○, cells that received 10% foetal calf serum for the final 30 min of incubation.

phosphodiesterase activity is shown to be reduced threefold at this time (Fig. 1c). Fig. 1b demonstrates an increase in total cyclic AMP phosphodiesterase activity after the addition of 10% serum. We have found that the cyclic AMP phosphodiesterase activity at zero time measured at 1 μM cyclic AMP may vary depending on the time of incubation in the 1% serum media and the serum lot used. An average increase of 3.5-fold (four experiments having a range of 2.8–5.3-fold increase) was seen, however, after addition of 10% serum.
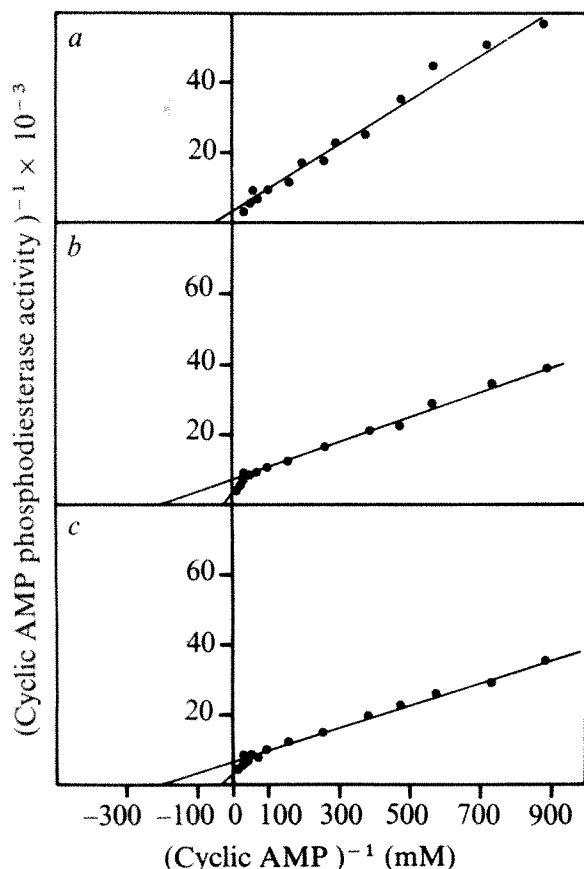
Fig. 3 Cells were prepared as described for the experiments in Fig. 2 with the exception that some cultures were incubated in cycloheximide (0.2 mM) for three hours before the addition of 10% serum. *a*, Cells in MEM with 1% serum and cycloheximide for the final 3 h incubation; *b*, additional 30 min incubation in 10% serum with no cycloheximide; *c*, same as in *a* except that after a 3 h preincubation in cycloheximide 10% serum was added to the cultures for the final 30 min.

Kinetic analysis of cell homogenates showed that the cyclic AMP phosphodiesterase of the cells incubated in medium containing 1% serum had an apparent K_m of 15 μM cyclic AMP (Fig. 2). Cyclic AMP phosphodiesterase activity from the cells which had been incubated for an additional 30 min in medium with 10% serum, however, showed activity with anomalous kinetic behaviour and apparent K_m values of 3 and 20 μM cyclic AMP. A 5-fold decrease in the K_m of cyclic AMP phosphodiesterase was evident within 30 min after the addition of serum to quiescent BHK cells.

To determine whether protein synthesis is required to obtain a serum-induced cyclic AMP phosphodiesterase
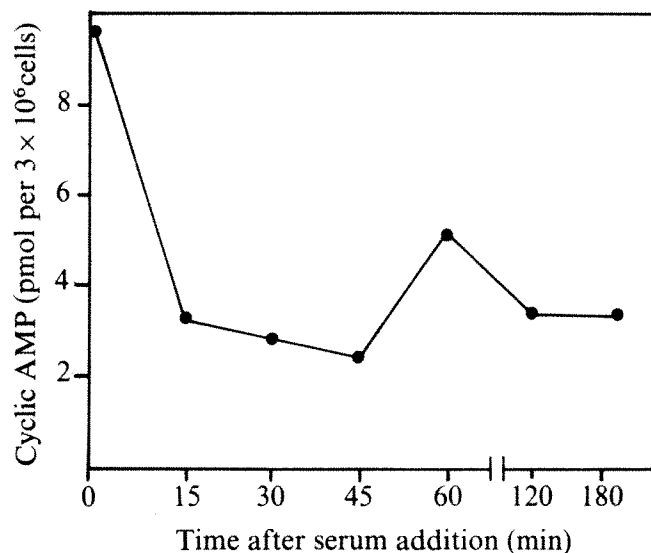
Fig. 4 Culture dishes (1 × 10⁶ cells per plate) were incubated for 36 h. At zero time 10% serum was added to the plates and at the indicated times cells were collected for cyclic AMP determinations. To determine cyclic AMP content, the medium was poured from the plate and 0.05 N perchloric acid was added before scraping the cells from the plates. Cyclic nucleotide concentration was analysed by the radioimmunoassay according to Steiner *et al.*[11] as modified by Thompson and Williams[12].

activity with a greater apparent affinity for cyclic AMP, BHK cells were incubated as described for Fig. 2, except that during the 3 h before the addition of 10% serum either cycloheximide (0.2 mM) or a vehicle control was added. Following this treatment, cultures were either retained in 1% serum or had 10% serum added for the final 30 min incubation. The Lineweaver–Burk analysis[6] of the homogenate activities from cells undergoing these treatments is shown in Fig. 3. The phosphodiesterase activities of the cells maintained in 1% serum in the presence of cycloheximide have a lower affinity (higher apparent K_m) for cyclic AMP than do the cells receiving 10% serum in the presence of cycloheximide (comparison of Fig. 3a with b and c). Analysis of the quiescent cells receiving additional serum did not reveal any differences in kinetic profiles between the vehicle control cells and those that received cycloheximide (Fig. 3b and c). These data indicate that the rapid change in substrate affinity of the cyclic AMP phosphodiesterase activity seen in quiescent cells in response to growth-promoting concentrations of serum takes place in the absence of protein synthesis.

Cyclic AMP levels decrease rapidly following the addition of 10% serum to quiescent BHK cells. The decrease in cyclic AMP levels is evident within 15 min after addition of serum (Fig. 4) and correlates with the increase in activity of cyclic AMP phosphodiesterase measured at 1 μM cyclic AMP (Fig. 1). Schröder and Plagemann reported that serum contains a cyclic nucleotide phosphodiesterase[7]. We also detected phosphodiesterase activity in foetal calf serum but

the data suggest little if any contribution of this enzyme to our experimental results. First, the specific activity of the serum enzyme using micromolar substrate concentrations of cyclic AMP in the assay is not sufficient to account for the changes in cyclic AMP phosphodiesterase activity seen in BHK cells after serum addition unless the cells did selectively extract all of the serum enzyme activity. Second, the serum enzyme can also hydrolyse cyclic GMP but we found a decrease in cyclic GMP phosphodiesterase activity in response to the addition of serum. Finally, previous studies with surface cultures of confluent BHK cells demonstrated a reduction in total phosphodiesterase activity when these cells were diluted to a lower density in 10% foetal calf serum[8].

These data support our hypothesis that substrate affinities of the cyclic AMP phosphodiesterase activities are altered as a response to certain conditions that affect cell growth[8]. These same conditions result in a concomitant, rapid and transient change in intracellular cyclic nucleotide levels. Thus, the substrate affinity as well as the amount of phosphodiesterase could be involved in regulation of cyclic nucleotide concentrations. Alterations in substrate affinity of the enzyme may be an important early step preceding cyclic nucleotide changes and altered growth states.

W. J. Pledger
W. J. Thompson
S. J. Strada

The University of Texas Medical School at Houston,
Program in Pharmacology,
Houston, Texas 77025

1 Temin, H. M., Pierson, R. W., and Dulab, N. C., in *Growth, Nutrition and Metabolism of Cells in Culture* (edit. by Rothblat, G. H., and Cristofals, V. J.), 50–82 (Academic London, 1972).
2 Sheppard, J. R., *Nature new Biol.*, **236**, 14–16 (1972).
3 Seifert, W., and Paul, D., *Nature new Biol.*, **240**, 281–283 (1972).
4 Seifert, W. E., and Rudland, P. S., *Nature*, **248**, 138–140 (1974).
5 Rudland, P. S., Seeley, M., and Seifert, W., *Nature*, **251**, 417–419 (1974).
6 Lineweaver, H., and Burk, D., *J. Am. chem. Soc.*, **56**, 658–666 (1934).
7 Schröder, J., and Plagemann, P. G. W., *J. natn. Cancer Inst.*, **46**, 423–429 (1971).
8 Pledger, W. J., Thompson, W. J., and Strada, S. J., *J. Cyclic Nucl. Res.* (in the press).
9 Thompson, W. J., and Appleman, M. M., *Biochemistry*, **10**, 311–316 (1971).
10 Thompson, W. J., and Appleman, M. M., *J. biol. Chem.*, **246**, 3145–3150 (1971).
11 Steiner, A. L., Parker, C. W., and Kipnis, D. M., *J. biol. Chem.*, **247**, 1106–1113 (1972).
12 Thompson, W. J., and Williams, R. H., *Archs biochem. Biophys.*, **165**, 468–477 (1974).

Evidence for immunoreactive somatostatin in the endocrine cells of human foetal pancreas

Somatostatin has been identified as a hypothalamic factor inhibiting the release of growth hormone[1,2] by acting directly at the level of adenohypophysial cells. It also acts on pancreatic endocrine cells[3,4] to inhibit the secretion of glucagon and insulin. Immunohistochemistry has localised somatostatin in the median eminence[5-7] as well as in discrete cells of the Langerhans islets of several species[8,9]. The origin of pancreatic immunoreactive somatostatin poses a problem. It has been demonstrated that neither nerve fibres, nor nerve endings with immunoreactive somatostatin are found in the adult pancreas[9]. Therefore it has been suggested that the somatostatin detected in the pancreas does not

result from absorption by these cells of somatostatin synthesised elsewhere (in the central nervous system for instance) but is produced by the pancreatic cells (probably A1 cells) themselves[9]. The results reported here show the presence of immunoreactive somatostatin in the pancreas of normal human foetuses and one totally anencephalic human foetus.

The pancreases were removed from 6 to 24-week-old human foetuses obtained immediately after legal abortion, or from deceased premature infants. After fixation for 2 or 3 d in Bouin–Holland fluid, without acetic acid, and with 5% saturated Hg sublimate added, the tissues were carefully washed in water, then dehydrated and embedded in
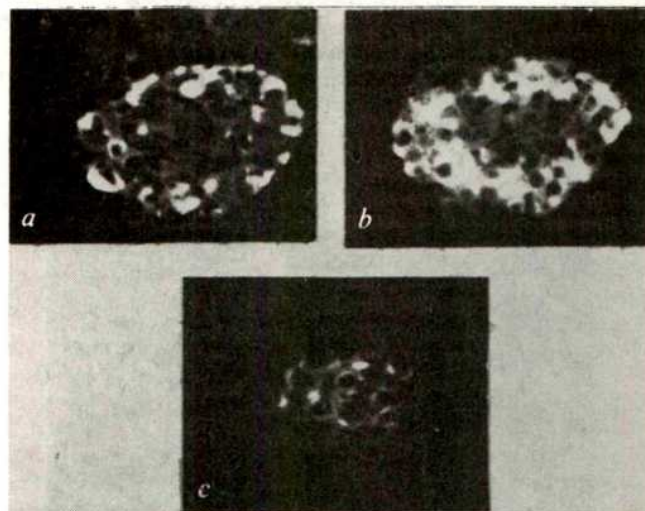

Fig. 1 Serial sections of a single islet of normal human 16-week-old foetus after application of *a*, anti-somatostatin; *b*, anti-glucagon; and *c*, anti-insulin.

paraffin. Sections (5 μm) were used after fixing on to glass slides with 0.5% aqueous gelatin.

Hormones were detected in the cells using immunofluorescence. Anti-somatin[9], anti-glucagon[9] and anti-insulin were used as antisera. Immunofluorescence was detected by the indirect method with the aid of sheep anti-rabbit-γ-globulins conjugated with fluorescein isothiocyanate (Pasteur Institute, Paris). In all cases, the antisera were saturated with the particular proteins used in the coupling of antigen. The specificity of the immunofluorescent reaction was ascertained by studying the inhibition of the reaction by homologous and heterologous antigens (somatostatin, glucagon, insulin).

Figure 1 shows the reaction of the normal 16-week-old foetus endocrine cells with the three antisera. Three types of cells were distinguished—somatostatin-containing cells and glucagon-containing cells were often located in the islet periphery, insulin-containing cells, in the islet centre (Fig. 1*a*, *b* and *c*). Different cells reacted with each serum.

No immunofluorescent reaction was seen with antisomatostatin and anti-glucagon before 10–11 weeks of gestational age and before 12 weeks of age with antiinsulin. At the third month, the fluorescent cells are located close by or in the wall of branching ducts embedded in connective tissue of the pancreatic parenchyma. After this time, they are located in islets.

Figure 2 shows the endocrine pancreatic cells of an anencephalic 7.5-month-old foetus reacting to antisomatostatin. Immunofluorescence was also observed with anti-glucagon and anti-insulin. The cells were smaller than in the normal foetus. A fluorescent reaction was never observed

with anti-somatostatin either in nerve fibres or in nerve endings throughout the pancreas.

In the human foetal pancreas, inhibition of the immuno-fluorescent reaction was seen only after adding somatostatin in a concentration equal to 400 $\mu g\,ml^{-1}$ of undiluted antiserum. Glucagon and insulin do not interfere with the immunocytological binding of somatostatin antiserum. Similarly, only insulin inhibits the immunofluorescence reaction caused by insulin antiserum, and only glucagon inhibits the immunofluorescence reaction caused by glucagon antiserum. No reaction was obtained using normal rabbit serum in place of the antisera.

It has been demonstrated and reported elsewhere[7,9], that the anti-somatostatin serum used here does not react with any of the following peptides—LH-RH, TSH-RH, ocytocine, lysine vasopressin, neurophysin, glucagon, insulin, secretin and the tetrapeptide Thr–Phe–Thr–Ser, common to somatostatin, glucagon and insulin. These data and the study of inhibition of the immunocytological reaction show that the fluorescence is most certainly attributable to the presence of somatostatin in the pancreatic cells. Therefore it

Fig. 2 Anencephalic human 7.5-month-old foetus pancreas. Cells containing immunoreactive somatostatin in two adjacent islets were detected by anti-somatostatin.

may be concluded that human foetal pancreas somatostatin occurs by 11 weeks of gestation.

The presence of somatostatin-containing cells in the islets of normal human foetal pancreas agrees with the recent observation of a somatostatin activity detected by bioassay in aqueous extract of foetal rat pancreas[10]. Also, the presence of glucagon-containing cells at 10–11 weeks of gestation and of insulin-containing cells at 12 weeks is consistent with the presence of the same hormone as demonstrated by radioimmunoassay for glucagon[11,12] or insulin[13,14] and by morphological techniques[15,16], all of these in human foetal pancreas. It has been suggested that the somatostatin-containing cells were the A1 cells[9]. Indeed, these cells have been identified in the human foetal pancreas from about the third month[16].

Whether pancreatic somatostatin participates in some mechanism involved in the central control of glucagon and/or insulin secretion in the human pancreas during intra-uterine life is still unknown.

The data reported here demonstrate that somatostatin is not an exclusively hypothalamic hypophysiotrophic peptide and may be synthesised elsewhere.

P. M. DUBOIS
C. PAULIN

*Laboratoire d'Histologie et Embryologie,
UER Médicale Lyon Sud-Ouest,
BP n° 12, 69600 Oullins, France*

R. ASSAN

*Laboratoire de Diabétologie,
Hôtel Dieu, 75005 Paris, France*

M. P. DUBOIS

*Laboratoire de Physiologie de la Reproduction,
INRA, 37380 Nouzilly, France*

1 Vale, W., Brazeau, P., Grant, G., Nussey, A., Burgus, R., Rivier, J., Ling, N., and Guillemin, R., *C.r. hebd. Séanc. Acad. Sci., Paris*, 275D, 2913–2915 (1972).
2 Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J., and Guillemin, R., *Science*, 179, 77–79 (1973).
3 Koerker, D. J., Ruch, W., Chideckel, E., Palmer, J., Goodner, C. J., Ensinck, J., and Gale, C. C., *Science*, 184, 482–484 (1974).
4 Gerich, J., Lovinger, R., Grodsky, G., *Endocrinology*, 94, 190 (1974).
5 Pelletier, G., Labrie, F., Arimura, A., Schally, A. V., *Am. J. Anat.*, 140, 445–450 (1974).
6 Hoekfelt, T., Effendic, S., Johansson, O., Luft, R., and Arimura, A., *Brain Res.*, 80, 165–169 (1974).
7 Dubois, P. M., Barry, J., and Leonardelli, J., *C.r. hebd. Séanc. Acad. Sci. Paris*, 279D, 1899–1902 (1974).
8 Luft, R., Effendic, S., Hökfelt, T., Johansson, O., and Arimura, A., *Med. Biol.*, 52, 428–430 (1974).
9 Dubois, M. P., *Proc. natn. Acad. Sci. U.S.A.*, 72, 1340–1343 (1975).
10 Vale, W., and Guillemin, R., *Rec. Prog. Horm. Res.* (in the press).
11 Assan, R., and Boillot, J., in *Metabolic processes in the fetus and newborn infant* (edit. by Jonxis, J. H. P., Visser, U. K. A., and Troelstra, J. A.), 210–219 (Stenfert Kroese, Leiden, 1971).
12 Scharffer, L. D., Wilder, M. L., and Williams, R. H., *Proc. Soc. exp. Biol. Med.*, 143, 314 (1973).
13 Steinke, J., and Driscoll, S. G., *Diabetes*, 14, 573–578 (1965).
14 Grumbach, M. M., and Kaplan, S. L., in *Foetal and Neonatal Physiology*, 462–487 (Cambridge Univ. Press, Cambridge, 1973).
15 Like, A. A., and Orci, L., *Diabetes*, 21, 511–534 (1972).
16 Bjorkman, N., Hellerström, C., Hellman, B., and Peterson, B., *Z. Zellforsch.*, 72, 425–445 (1966).

Scrapie incubation time can exceed natural lifespan

IN mice injected with mouse-passaged scrapie, the earliest rise in titre of agent is found in organs of the lymphoreticular system —irrespective of whether the injection is intracerebral or by a peripheral route[1-4]. The titre of agent in the spleen, for example, can rise fairly quickly to a moderate plateau level and subsequently starts to increase progressively in the brain, where it reaches, during the clinical phase, a greater concentration than elsewhere. These generalisations are based on work in various laboratories with agent strain–mouse strain combinations having relatively short incubation periods in the range 150–300 d after injection of high doses of agent. Evidence of replication in the spleen is therefore an indication that the animal will eventually develop scrapie if it lives long enough.

The detailed titre levels and time sequences for the start of replication in the spleen depend on the strain and dose of agent, on the genotype of the mouse and its age at injection, and on the route of injection[5,6]. In particular the gene *sinc* (alleles, s7 and p7) is important in controlling agent replication and the details vary widely for different strains of agent[7]. It has been shown with ME7 agent, that in mice homozygous for p7 there is a delay of about 4 weeks before replication starts in the spleen after injection of 10^4 C57BL intracerebral LD_{50} units of agent whereas in *sinc*[s7] mice the delay, if any, does not exceed 3 d; the respective incubation periods in these examples are approximately 300 d and 160 d.

Table 1 Number of infectious units of agent in spleen and incidence of clinical scrapie in C57BL mice injected intraperitoneally with various concentrations of 22A and, for comparison, incubation periods and incidences for the same doses given intracerebrally

Dilution of VM 22A-infected brain*	22A dose† injected	Intraperitoneal injection						Intracerebral injection	
		22A concentration† per mg spleen at various intervals after injection				Scrapie incidence	Incubation period (d ± s.e.) or observation period (d)	Scrapie incidence	Incubation period (d ± s.e.)
		60 d	100 d	300 d	600 d				
10^{-1}	20,000	ND	ND	ND	ND	7/7	562 ± 15	4/4	452 ± 2
10^{-2}	800	0	0	25–50	25–50	0/4†	640–736	18/18	479 ± 4
10^{-3}	80	0	0	0	25–50	0/3	639–841	10/10	495 ± 7
10^{-4}	8	0	0	0	0	0/3	Discontinued	4/6	548 ± 13
10^{-5}	⩾1	0	0	0	0	0/4	at 630	3/7	571 ± 48
10^{-6}	0	ND	ND	ND	ND	ND	ND	0/6	—

* 10^{-1} inoculum, 500g, 10 min supernatant; others, 2,000g, 15 min, supernatant.
†VM intracerebal LD_{50} units of 22A; estimated concentrations were within the ranges given.
‡See text.
ND, Not determined.

For each agent strain–mouse strain combination, incubation period increases as dose is decreased and is longer after intraperitoneal than intracerebral injection. Even with intracerebral injections it is difficult to determine the titration endpoint for some agent–mouse combinations because the incubation period at the lower doses is of the same order as the lifespan of the mouse. Thus, with 22A agent in C57BL mice (sinc^{s7}) individual incubation periods range from about 450 d with 10^{-1} 22A-infected brain homogenates to about 670 d with the 10^{-5} dilution, and many of the apparent survivors are senile by this stage (Table 1: estimated $\log_{10}$ C57BL intracerebral LD_{50} Reed–Muench titre is -4.6). In these senile mice there is an element of uncertainty in the histological criteria for deciding whether a mouse is dying of scrapie or of old age because there is an overlap in the range of histological lesions of scrapie and those caused by brain ageing[8]. Assay of agent in the spleen provides a potential means of resolving the dilemma of whether, in the type of example given, titre is being underestimated by cases of scrapie in the 'next higher dilution' not having the opportunity to appear during the normal lifetime. To assay this efficiently would require using another genotype of mouse in which the particular agent replicates much more rapidly, which in the case of 22A agent would be VM mice (sinc^{p7}). The following experiment shows that this approach is practicable in even more extreme circumstances.

C57BL mice were injected intraperitoneally at weaning with various saline dilutions of 22A-infected VM brain homogenate. One female and one male mouse in each dilution from 10^{-2} to 10^{-5} was killed at 60, 100, 300 and 600 d after injection and the spleens removed for assay. The assay involved intracerebral injection of groups of weanling VM mice with 10^{-1} C57BL spleen homogenates, which were not centrifuged, and estimating the titre from the incubation period[9]. The results for the female spleens are shown in Table 1; preliminary results (not given) for male spleens show the same general pattern.

In addition, some C57BL mice in each dilution group were observed for signs of scrapie: all the mice given the 10^{-1} dose developed typical symptoms and lesions but in the lower dilutions none developed unequivocal signs. When individuals were senile or showing equivocal signs of chronic neurological illness they were killed and their brains examined histologically, as were the brains of all those killed earlier for spleen assay. None of the mice in the 10^{-2} to 10^{-5} groups had typical scrapie lesions, but one of the four mice in the 10^{-2} group (killed 709 d after injection) presented some difficulties in deciding that the lesions were probably caused by ageing—the spleen of this mouse had 50–100 VM intracerebral LD_{50} units 22A mg^{-1}. The only other mouse brain in the whole experiment which presented such difficulties was from the 10^{-2} group male killed at 600 d for spleen removal. It is possible therefore that scrapie was partly responsible for the equivocal symptoms and lesions seen

in a minority of the 10^{-2} group. If only clinical cases of scrapie are used as the basis for calculation, the estimated intracerebral titre is 1,000 times higher than that for the intraperitoneal route but this reduces to about 50 times higher if allowance is made for animals with agent in the spleen.

The results of the spleen assays form a clear and consistent pattern, with agent only being detectable in the two higher-dose groups and being detected earlier in the 10^{-2} group than the 10^{-3} group, but even then no agent was detected in the spleen until at least 100 d after injection. It is not yet known whether agent is present in the spleen for a few days immediately after injection and then cleared, but it seems unlikely that agent first recovered at 300 d is part of the original inoculum, slowly accumulating there. The reason for this long absence from the spleen is unknown and it could even be an artefact of insensitive assay systems. The crucial point will be whether agent can be detected elsewhere in the body during this interval and in which organs. Until these points are settled it would be quite inappropriate[4] to refer to this interval as an 'eclipse phase' and without direct evidence it should not be assumed that this, or other, conventional virological categories apply to scrapie[10].

If incubation can exceed lifespan with scrapie there seems to be no reason why this will not occur with other members of this group of diseases. It is therefore possible that a greater number of individuals are infected with one of the agents causing transmissible encephalopathies or dementias, such as Creutzfeldt–Jakob disease in man, than the number of cases which occur. Such individuals are, presumably, potential sources of infection, especially where tissue transplantation is involved[11].

A. G. DICKINSON
H. FRASER
G. W. OUTRAM

*ARC Animal Breeding Research Organisation,
Edinburgh, EH9 3JQ and
Moredun Research Institute,
Edinburgh EH17 7JH, UK*

1 Eklund, C. M., Kennedy, R. C., and Hadlow, W. J., *J. infect. Dis.*, 117, 15 (1967).
2 Dickinson, A. G., and Fraser, H., *J. comp. Path.*, 79, 363 (1969).
3 Clarke, M. C., and Haig, D. A., *Res. vet. Sci.*, 12, 195 (1971).
4 Hunter, G. D., Kimberlin, R. H., and Millson, G. C., *Nature new Biol.*, 235, 31 (1972).
5 Dickinson, A. G., *Genetics*, 79, 387 (1975).
6 Outram, G. W., Dickinson, A. G., and Fraser, H., *Nature*, 241, 536 (1973).
7 Dickinson, A. G., and Meikle, V. M. H., *Molec. gen. Genet.*, 121, 73 (1971).
8 Fraser, H., *Proc. sixth Int. Congr. Neuropath.*, 897 (Masson, Paris, 1970).
9 Dickinson, A. G., Fraser, H., Meikle, V. M. H., and Outram, G. W., *Nature new Biol.*, 237, 244 (1972).
10 Outram, G. W., in *Slow Virus Diseases*, ch. 14 (edit. by Kimberlin, R. H.) (Elsevier, Amsterdam, in the press).
11 Duffy, P., Wolf, J., Collins, G., De Voe, A. G., Streeten, B., and Cowen, D., *New Engl. J. Med.*, 290, 692 (1974).

Translational control in the mealworm, *Tenebrio molitor*

ONE of the best known examples of translational control in a eukaryotic system comes from studies on the mealworm, *Tenebrio molitor,* in which control of the synthesis of adult cuticular proteins at the translational level has been proposed[1-7]. From experiments using actinomycin D, it was concluded that the mRNAs for some of the adult cuticular proteins were synthesised by the first day of pupation[1]. To translate these mRNAs *in vitro* on first day polysomes, however, the tRNA and enzyme from 6 to 7 d pupae were required[2-4]. Ilan *et al.* gave further evidence for a leucine tRNA which seemed to be controlling the translation of the mRNAs and the synthesis of which seemd to be in the control of juvenile homone[3-7]. But our own studies of *Tenebrio,* reported here, lead us to question this translational control model.

We decided to look at changes of tRNAs during the development of *Tenebrio* and compare them with changes observed during the development of *Drosophila*[8-10]. Transfer RNA and aminoacyl-tRNA synthetases were extracted by the methods described previously[8]. Aminoacylation conditions were established that would give plateau levels of charging by 10 min. We found the conditions used by Ilan *et al.*[4] for aminoacylating leucine tRNAs gave a very slow rate of charging chiefly because of the relatively low affinity of the *Tenebrio* leucyl-tRNA synthetase for leucine, the monovalent cation requirement and the ATP–Mg^{2+} ratio used. We found the K_m of the enzyme for leucine to be 20 μM. This compares with an even higher K_m (200 μM) reported by Ilan and Ilan[7].

Using the improved aminoacylating conditions, the amounts of the leucine tRNAs as well as other tRNAs were determined in both early pupae and late pupae (pharate adults) (Table 1). When compared with either phenylalanine or tyrosine tRNAs, there was no significant change in the total acceptance of leucine by tRNA preparations from these two developmental stages. Even though there was not the expected net increase in the acceptance of leucine there could have been a new leucine tRNA formed with a concomitant decrease in the other isoaccepting species. To examine this possibility, ^{14}C-leucyl-tRNAs from both early and late pupae were chromatographed on reversed-phase 5 columns by methods described previously[8]. The profiles of the leucyl-tRNAs from larvae, first-day pupae, last-day pupae and adults aminoacylated with a last-day pupal aminoacyl-tRNA synthetase preparation, were

virtually identical (Fig. 1). The same result was obtained using last-day pupal microsomal enzyme[4] as well as larval, first-day pupal and adult aminoacyl-tRNA synthetase preparations. This was surprising in view of the results of Ilan and Ilan that indicated the synthesis of a new leucine tRNA[5,7].

We, therefore, went back to the conditions used by Ilan *et al.* for the aminoacylation of the leucine tRNAs (Fig. 2).
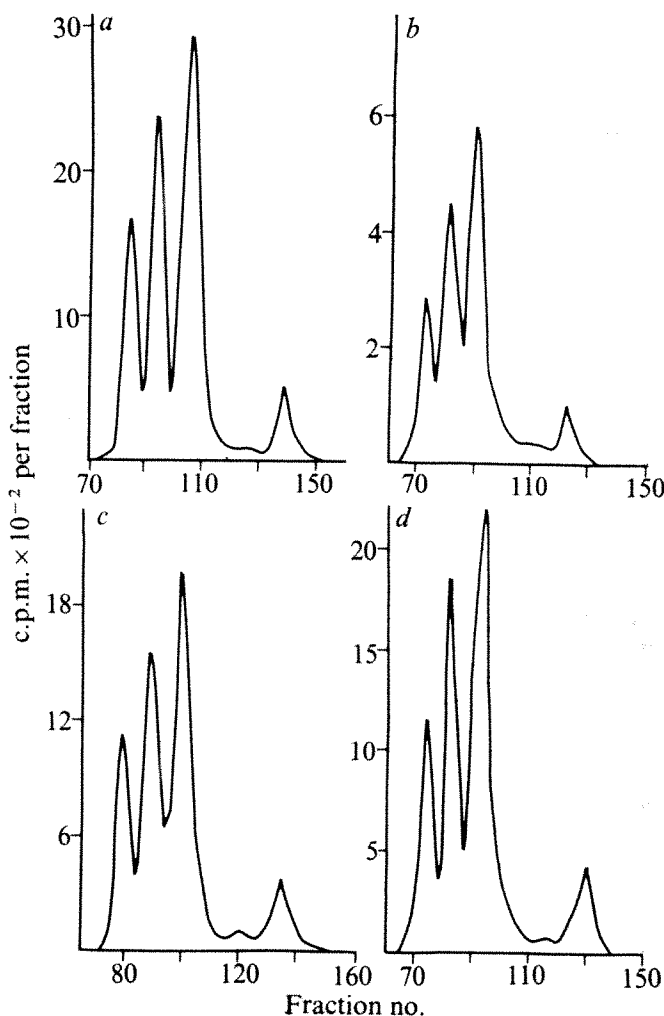

Fig. 1 Chromatography of ^{14}C-leucyl-tRNAs from different developmental stages of *Tenebrio.* Aminoacylation was performed as described in the legend of Table 1 using last-day pupal aminoacyl-tRNA synthetase preparations. Chromatography of leucyl-tRNAs on RPC-5 columns was performed as described previously[8]. *a,* Larval; *b,* first-day pupal; *c,* last-day pupal; *d,* adult.

**Table 1** Acceptance of amino acids by first-day and last-day pupal tRNAs

Amino acid	pmol amino acid accepted per A_{260} unit of total tRNA		First-day/last-day
	First-day pupal	Last-day pupal	
Leucine	55.3	51.5	1.07
Phenylalanine	26.3	25.4	1.02
Tyrosine	24.7	22.7	1.09

Transfer RNA and aminoacyl-tRNA synthetases were prepared as described previously[8]. Last-day pupal aminoacyl-tRNA synthetase was used. The aminoacylation conditions for leucine tRNAs were 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 8 mM ATP, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 μM ^{14}C-leucine, 1 mg ml^{-1} enzyme preparation and 0.5 mg ml^{-1} tRNA. The conditions for the aminoacylation of phenylalanine and tyrosine tRNAs were the same except 50 mM KCl, 4 mM ATP, 10 mM $MgCl_2$ and 25 μM ^{14}C amino acid were used.

The plateau level observed clearly represents incomplete charging and could not be used to estimate the amount of leucine tRNA present. We also chromatographed the ^{14}C-leucyl-tRNA obtained in these conditions on RPC-5 columns (Fig. 3). It is apparent that peak 1 and peak 2 are poorly charged in relation to peak 3. Similar results were obtained when aminoacylation was performed in conditions where the amino acid or enzyme was limiting.

These results immediately suggested an explanation for the differences between our results and those of Ilan *et al.*[3-7]. As aminoacylation in suboptimal conditions can give rise to different isoacceptor profiles, any comparison of tRNAs from different developmental stages can only be performed when rapid complete charging is achieved. The charging conditions used by Ilan *et al.*[4] produced rates that
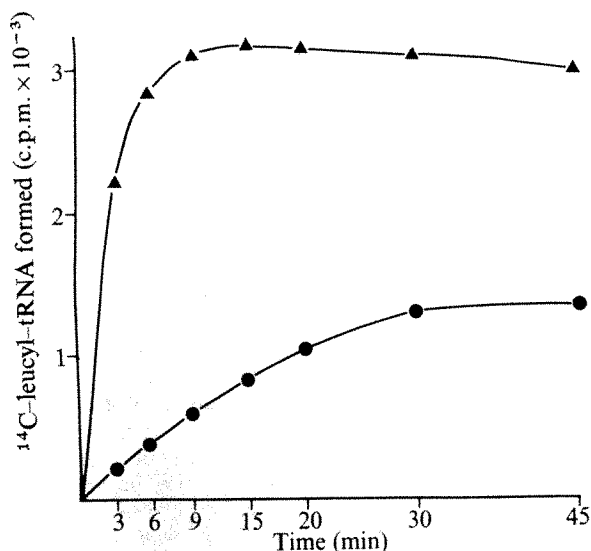
Fig. 2 Rates of aminoacylation of larval tRNA with leucine under varying conditions. ▲, Aminoacylation in the conditions described in the legend of Table 1; ●, aminoacylation conditions were similar to those described by Ilan *et al.*[4]; 50 mM imidazole, *p*H 7.0, 10 mM MgCl$_2$, 4 mM ATP, and 4 μM ^{14}C-leucine.

gave plateau levels only after about 50 min, and the results presented here indicate that charging under such conditions is incomplete especially for certain leucine isoaccepting tRNAs. Another problem with any analysis of aminoacyl-tRNAs using dual labels[5,7] is that the concentration of the differently labelled amino acids in both charging mixtures must be identical and the comparison made reciprocally to avoid isotope artefacts[11]. This is especially true when the labelled amino acid substrates are used at concentrations that are well below the K_m value of the aminoacyl-tRNA synthetase. The observed differential charging rates could produce apparent control during the translation of mRNAs *in vitro*, due not to lack of a particular tRNA but to incomplete aminoacylation.

Another weakness in the evidence for translational control of adult cuticular protein relates to the amino acid

Fig. 3 Chromatography of larval ^{14}C-leucyl-tRNAs aminoacylated in different conditions. *a*, Aminoacylated in the conditions described in the legend of Table 1; *b*, aminoacylated in conditions similar to those used by Ilan *et al.*[4], and described in the legend of Fig. 2.
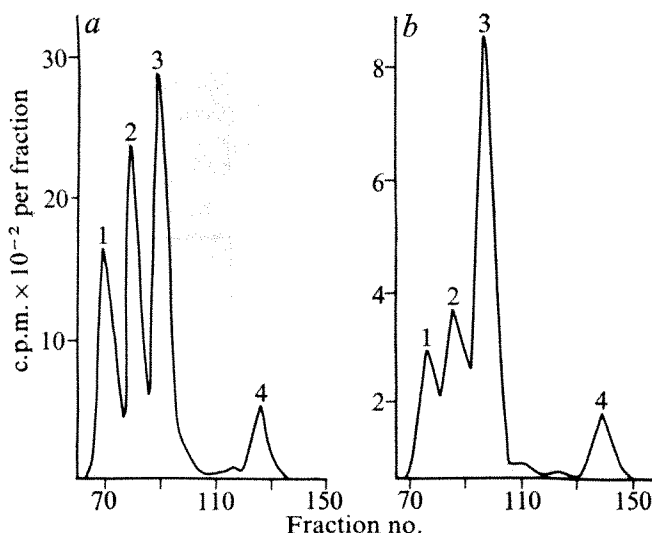
composition of these cuticular proteins. It has been claimed by Ilan *et al.*[4,6], apparently on the basis of published analyses on the beetle *Agrianome spinicollis*[13], that adult *Tenebrio* cuticles are high in tyrosine (20%) whereas pupal cuticles have 'low tyrosine'. This difference was used as an index of the synthesis of adult cuticular protein *in vitro*[4]. It has been shown by Anderson *et al.*[12] that the adult cuticle has in fact less tyrosine (4.6%) than pupal cuticle (9%). This, therefore, invalidates the use of high tyrosine content as an index of adult cuticular protein synthesis.

The ability of actinomycin D to mimic the action of juvenile hormone suggests control at the transcriptional rather than the translational level[14–16]. Our results would support this in that we have found no evidence for a novel leucine tRNA that might control the translation of preformed mRNA for adult cuticular protein. We feel that the suboptimal conditions used to aminoacylate the leucine tRNAs, coupled with the differential rates of charging of the leucine isoacceptor tRNAs, explains much of the data that originally led Ilan *et al.*[4] to suggest a translational control mechanism.

We thank Dr G R. Wyatt for reading the manuscript. The work was supported by the National Research Council of Canada and the Rockefeller Foundation.

NORMAN J. LASSAM
HARVEY LERER
BRADLEY N. WHITE

Department of Biology,
Queen's University,
Kingston, Ontario, Canada

1 Ilan, J., Ilan, J., and Quastel, J. H., *Biochem. J.*, 100, 441–447 (1966).
2 Ilan, J., *J. biol. Chem.*, 243, 5859–5866 (1968).
3 Ilan, J., *Cold Spring Harb. Symp. quant. Biol.*, 34, 787–791 (1969).
4 Ilan, J., Ilan, J., and Patel, N., *J. biol. Chem.*, 245, 1275–1281 (1970).
5 Ilan, J., and Ilan, J., in *The Physiology of Insecta* (edit. by Rockstein, M.), 4, 355–422 (Academic, New York, 1974).
6 Ilan, J., Ilan, J., and Patel, N. G., in *Insect Juvenile Hormones Chemistry and Action* (edit. by Menn, J. J., and Beroza, M.), 43–68 (Academic, New York, 1972).
7 Ilan, J., and Ilan, J., *Devl Biol.*, 42, 64–74, (1975).
8 White, B. N., Tener, G. M., Holden, J., and Suzuki, D. T., *J. molec. Biol.*, 74, 635–651 (1973).
9 White, B. N., Tener, G. M., Holden, J., and Suzuki, D. T., *Devl Biol.*, 33, 185–195 (1973).
10 Grigliatti, T. A., *et al.*, *Cold Spring Harb. Symp. quant. Biol.*, 38, 461–474 (1974).
11 Waters, L. D., and Novelli, D. D., in *Methods in Enzymology* (edit. by Grossman, L., and Moldave, K.), 20, 39–55 (1971).
12 Anderson, S. O., Chase, A. M., and Willis, J. H., *Insect Biochem.*, 3, 171–180 (1973).
13 Hackman, R. H., and Goldberg, M., *J. Insect Physiol.*, 2, 221–231 (1958).
14 Chase, A. M., *Nature*, 215, 1516–1517 (1967).
15 Chase, A. M., *J. Insect Physiol.*, 16, 865–884 (1970).
16 Socha, R., and Sehnal, F., *J. Insect Physiol.*, 18, 317–337 (1972).

Extraordinary pigment composition of a prokaryotic alga

WE report an anomalous unicellular alga with a prokaryotic cellular organisation like that of a blue-green alga, but with a pigment composition characteristic of green algae and higher plants.

Synechocystis didemni Lewin is a unicellular marine alga so far found only associated with colonial ascidians (*Didemnum* spp.) on the coasts of Baja California, Mexico[1]. Under the light microscope it looks like a blue-green alga, and by electron microscopy of thin sections we have confirmed that the cellular organisation is prokaryotic, without membrane-limited nucleus, plastids or mitochondria (ref. 2 and R. A. L. and M. Schulz-Baldes, unpublished). Typically, blue-green algae—photosynthetic prokaryotes which can evolve oxygen when illuminated—contain at least one red or blue bilin pigment (phycoerythrin or phycocyanin) and only one chlorophyll, *a*, *S. didemni*, however, is leaf-green in colour, apparently
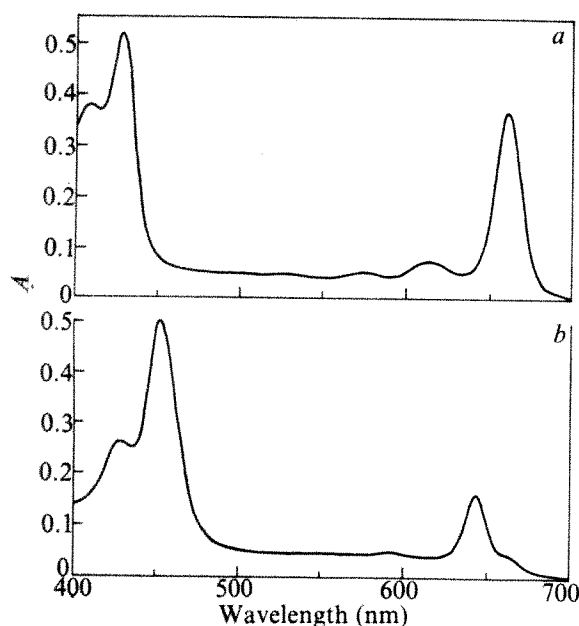
Fig. 1 Spectral absorption curves of solutions of *S. didemni* chlorophylls in ether, provisionally identified as *a* and *b*. For details of extraction and separation methods, see Table 2.

lacks bilin pigments, and contains two chlorophylls, *a* and *b*— features characteristic of green algae and land plants. Preliminary evidence for this unique combination of a prokaryotic organisation with a pigment complement so far only known in eukaryotic plants has been published elsewhere[2]. We offer here confirmation of the presence of chlorophylls *a* and *b*, together with other lipid-soluble (but no water-soluble) pigments.

So far it has not proved possible to culture cells of *S. didemni*; consequently we had to analyse living material collected from the wild. In March 1975, didemnid colonies bearing *S. didemni* were gathered in a tropical lagoon on Isla S. José, Baja California from dimly illuminated mangrove roots (maximum light intensity around noon, 300–500 lx; water temperature about 20–25 °C). The surfaces were brushed as described[2], and the algal cells removed were concentrated by centrifugation. We collected three lots of algal cells, each of a few hundred milligrams (wet weight). The pigments from small subsamples were extracted and examined chromatographically on the day of collection; the rest of each sample was stored at −20 °C for careful examination in the laboratories of the Scripps Institution of Oceanography several days later. (There was no reason to believe that storage in this way effected any appreciable change in the pigment composition of the samples.)

After thawing, the cells were washed in water, and the supernatant, essentially colourless, was examined for possible traces of bilin pigments. An absorption spectrum revealed no

evidence for any pigmented material of this sort. The lipid-soluble pigments were then extracted and examined by thin-layer chromatography (TLC) and spectrophotometry (Fig. 1 and Tables 1 and 2).

The evidence for the existence of chlorophyll *b*, as well as *a*, in *S. didemni* seems unequivocal. This second green pigment cochromatographed with chlorophyll *b* from the chlorophyte *Codium fragile* and exhibited absorption maxima (Table 2) and band ratios typical for *b* (ref. 3). In the two samples examined the ratio *a:b* was found to be comparable with that of other algae which contain *b* (Table 1). The presence of *b* in our preparations could hardly be attributable to contamination of the samples by other algae, as microscopic examination revealed that the cells in our suspensions were almost all *S. didemni*, with no evident chlorophytes or euglenophytes. There was a small proportion (less than 1%) of diatoms or diatom debris, but these were mainly empty frustules, and only a few retained pigmented contents. The second chlorophyll of diatoms, *c*, has an R_f and other properties quite dissimilar from those of *b*. No *c* was detected in our material.

The predominant carotenoid in *S. didemni* is β carotene, representing some 65% of the total absorption attributable to yellow pigments. This proportion is higher than that normally encountered in chlorophytes (for example, 15% for *Chlorella pyrenoidosa*, which also has α carotene), but is close to that found in certain cyanophytes (for example, 46% for *Anacystis nidulans*[5], 63% for *Microcoleus vaginatus*[6]). The other carotenoids of *S. didemni* were presumably xanthophylls, perhaps including myxoxanthophyll. The amounts were insufficient for critical analysis, which must evidently await the availability of much larger quantities of cell material, if and when this can be obtained in culture.

Although it has been generally accepted that all photosynthetic cyanophytes (blue-green algae) possess bilin pigments, in an unidentified blue-green alga associated with ascidians of the Great Barrier Reef little or no phycocyanin or phycoerythrin was detected[7]. It has also been generally accepted that blue-green algae lack *b* (ref. 8), although spectral data from blue-green algal mats collected in Yellowstone Park indicated that about 15% of the chlorophyll was present as *b* (ref. 9). Since those algae had grown at temperatures above 48 °C, they could hardly have been contaminated by chlorophytes or euglenophytes, none of which has been reported to grow in such hot water. Among the predominantly filamentous algae Inman[9] noted the presence of *Synechococcus lividus* (Chroococcales) and of "a unicellular green alga, probably *Chlorella* sp.". From what we now know of such habitats we can be almost certain that the latter alga was *Cyanidium caldarium*, which does not contain *b* (ref. 10).

In the original description of the type species of the genus, *Synechocystis aeruginosa*[11], the cells were described as being of a blue-green colour. Drouet and Daily[12], who re-examined Sauvageau's material, reported that it contained cells which seemed to belong to the Chlorococcales (Chlorophyta); but it is hard to believe that Sauvageau could have mistaken a unicellular cyanophyte in this way. Further conclusions may only be drawn after examination of the pigments of an authentic

Table 1 Comparative pigment data for *S. didemni* and other algae

	Phycobilins (g per 10^{14} cells)	Chlorophylls		*a:b*	β carotene/ total carotenoids	Calculated from data in ref.
		a (g per 10^{14} cells)	*b* (g per 10^{14} cells)			
S. didemni (from white *Didemnum**)		95	22	4.36		
S. didemni (from grey *Didemnum**)		47	6.9	6.92		
Anacystis nidulans	1,300	60	0	—	0.65	5, 14, 15
Chlorella pyrenoidosa		40	7.6	5.25	0.46	4, 16, 17
Euglena gracilis		200	33.3	6.0	0.15	18, 19, 20
					0.11	

*The species could not be determined.
Cell counts were made with a Levy haemocytometer. Carotenoids and chlorophylls were estimated spectrophotometrically using standard extinction coefficients[3,13].

Table 2 R_f values and absorption maxima (nm) of *S. didemni* pigments

R_f on sucrose (TLC)	Colour	Acetone	Solvent Ethyl ether	n-Hexane	Identity
0.92	Yellow	425, 452, 480	—	420, 448, 475	β carotene*
0.72	Blue-green	430, 662	431, 662	—	Chlorophyll *a*
0.61	Orange	425, 451, 480	—	—	Unidentified xanthophyll
0.48	Yellow-green	457, 645	452, 642	—	Chlorophyll *b*
0.19	Yellow	440, 470	—	—	Unidentified xanthophyll
0.02	Orange	445	—	—	Unidentified xanthophyll

*Failed to separate from authentic β carotene (from *Daucus carota*) in a mixed chromatogram on Al_2O_3 + MgO developed with 6% ethyl acetate in n-hexane[22].

Pigments were extracted by sonicating the cells in a small volume of cold acetone. Cell residues were removed by centrifugation, and the pigments from the supernatant were transferred to ethyl ether by addition of cold, saturated saline. The ether extract was concentrated under N_2 and subjected to TLC on sucrose, using 1.3% n-propanol in ligroine (boiling point 63–75 °C)[21].

strain of *S. aeruginosa* (we are attempting to obtain a culture of this species from the Department of Genetics, University of Moscow), and of the pigments of other cyanophytes grown in natural conditions, notably those encountered by *S. didemni* in mangrove swamps and by other species which survive in hot springs.

In conclusion, we have confirmed that natural collections of *S. didemni* contain chlorophylls *a* and *b*, β carotene, at least three xanthophylls, and no demonstrable water-soluble phycobilin pigment. In these respects it seems to be unique among cyanophytes which have been examined to date.

We thank Dr F. T. Haxo for advice and guidance with the chromatography, and the Foundation for Ocean Research for making the RV Dolphin available for collecting material.

R. A. LEWIN
NANCY W. WITHERS

Scripps Institution of Oceanography,
University of California,
La Jolla, California 92037

1 Lewin, R. A., and Cheng, L., *Phycologia*, 14 (in the press).
2 Lewin, R. A., *Phycologia*, 14 (in the press).
3 Strain, H. H., Thomas, M. M., and Katz, J. J., *Biochim. biophys. Acta*, 75, 306 (1963).
4 Allen, M. B., Goodwin, T. W., and Phagpolngarm, S., *J. gen. Microbiol.*, 23, 93 (1960).
5 Halfen, L. N., and Francis, G. W., *Archs Mikrobiol.*, 81, 25 (1972).
6 Goodwin, T. W., *J. gen. Microbiol.*, 17, 467 (1957).
7 Newcomb, E. H., and Pugh, T. D., *Nature*, 253, 533 (1975).
8 Allen, M. B., French, C. S., and Brown, J. S., in *Comparative Biochemistry of Photoreactive Systems* (edit. by Allen, M. B.), 33 (Academic, London 1960).
9 Inman, O. L., *J. gen. Physiol.*, 23, 661 (1940).
10 Allen, M. B., *Archs Mikrobiol.*, 32, 270 (1959).
11 Sauvageau, C., *Bull. Soc. bot. France*. 39, 104 (1892).
12 Drouet, F., and Daily, W. A., *Butler Univ. Bot. Stud.*, 12, 218 (1956).
13 Davies, B. H., in *Chemistry and Biochemistry of Plant Pigments* (edit. by Goodwin, T. W.), 489 (Academic, London, 1965).
14 Evans, E. L., and Allen, M. M., *J. Bact.*, 113, 403 (1973).
15 Allen, M. M., *J. Bact.*, 96, 831 (1968).
16 Myers, J., and Graham, J. R., *Pl. Physiol.*, *Lancaster*, 48, 282 (1971).
17 Jørgensen, E. G., *Physiologia Pl.*, 22, 1307 (1969).
18 Brown, J. S., Alberte, R. S., Thornber, J. P., and French, C. S., *Carnegie Instn Yb.*, 694 (1974).
19 Holowinsky, A. W., and Schiff, J. A., *Pl. Physiol.*, *Lancaster*, 45, 339 (1970).
20 Krinsky, N. I., and Goldsmith, T. H., *Archs biochem. Biophys.*, 91, 271 (1960).
21 Jeffrey, S. W., *Biochim. biophys. Acta*, 162, 271 (1968).
22 Chapman, D. J., and Haxo, F. T., *J. Phycol.*, 2, 89 (1966).

Neuronal plasticity and recovery of function in a polyclad flatworm

NEURONAL recovery of function, although well known in the lower metazoa, has received comparatively little attention or critical investigation. We have been able to demonstrate not only recovery of function but also the recruitment of pathways mediating aspects of feeding behaviour in the marine polyclad flatworm, *Notoplana acticola*. Unlike some freshwater planarians which regenerate lost parts readily and may even grow new brains, polyclads have limited powers of regeneration and the brain seems to be essential for any repair which takes place[1]. Three aspects of feeding behaviour were utilised in this study. When a normal intact flatworm is presented with food (dead adult brine shrimp) at its posterior laterial margin, that part of the

margin in contact with the food is extended and grips the shrimp. This local response is followed by an ipsilateral turning and contraction which brings the anterior margin in contact with the food which it grips and then passes the shrimp towards the midventral mouth. After feeding on two or three shrimp, the worms are satiated and no longer respond to the presence of food. Both turning and satiation are altered when the brain is removed. Decerebrate animals do not turn, the local response is prolonged, and the food is passed directly to the mouth where it is swallowed. After the stomach is filled the worms continue to collect food which is piled under the mouth. This localised feeding response (LFR) continues to occur as long as food is presented. The presence of the brain is necessary to inhibit the LFR as well as to initiate turning and satiation.

If a cut is made from the midline to the margin right through the entire body, that portion of the animal ipsilateral to and posterior to the cut will act as if it is decerebrate. The contralateral side, however, will still turn and with distension of the stomach this side will ignore offered food. A single animal can then be used for both experiment and control.

Table 1 Recovery of turning behaviour component of feeding in *N. acticola*, following a cut across one side of the body severing the nerve plexus

Treatment	Experimental side			Control side		
	T(%)	LFR(%)	N	T(%)	LFR(%)	N
Normal response	100.0	0.0	51	100.0	0.0	51
Cut (experimental side)	4.3	95.7	92	92.3	7.7	91
Healed	94.9	5.1	99	97.9	2.1	94
Cut (control side)	79.8	20.2	84	0.0	100.0	90

N, Number of observations; T, turning reflex.

Ten animals were used and, except for the initial test, approximately ten responses from each side were obtained. Sides were tested alternately. Animals were kept in small fingerbowls, 11 cm diameter and containing approximately 2.5 cm seawater. The water was changed every 2 d but the inner surface of the bowl was not wiped clean. This is critical as animals will not feed until a layer of slime has been deposited on the surface of the container[2]. Dishes were not aerated and were maintained at ambient room temperature which fluctuated between 18 and 20 °C. With sufficient food animals can be kept indefinitely and will even grow. In each experiment 10 animals were used. Pairs were placed in separate dishes and allowed to acclimatise to the containers for 48 h before the start of the experiment. Individuals in a pair could be identified by size or slight colour differences.

Animals were tested for the turning response by being offered food on alternate sides (Table 1). Normal animals always turned to the side stimulated. During the course of the experiments described here food was always removed before it could be ingested. One side of each animal was cut (the experimental side) and the two sides were tested with food 20 h later. Nine of the ten animals now responded with LFR on the experimental side although one individual still turned in four of eleven tests. The control side turned in 92% of the tests and used LFR the other 8%. These animals were then retested 49 h after the cut had been made, by which time the edges seemed to have rejoined. When food was offered to the experimental side the animals turned 95% of the time. During these tests five LFRs were recorded from four of the animals. The recovery of turning behaviour

Table 2 Recovery and recruitment of feeding behaviour in *N. acticola*

Treatment	Experimental side			Control side		
	T(%)	LFR(%)	N	T(%)	LFR(%)	N
Normal response	100.0	0.0	100	—	—	—
Cut (experimental side)	5.6	94.4	84	100.0	0.0	91
Healed	92.1	7.9	98	97.9	1.1	98
Recut (exerimental side)	72.1	27.8	103	100.0	0.0	86

Ten animals were used in these experiments, approximately ten readings were obtained from each animal.

could be accounted for in either of three ways. Functional connections could have been re-established across the lesioned zone. Secondly, new or unused pathways across to the contralateral side could have been recruited. Finally, mechanical coupling across the wounded area might have elicited activity from stretch or other sensory receptors which could then evoke the turning response. Histological examination showed that the severed nerves had become reconnected (Fig. 1). To see whether contralateral pathways were being recruited, the animals were cut across the control half slightly anterior to the previous cut on the experimental side. As might be expected the control side responded with LFRs to each food presentation. Eight of the animals turned toward food presented to the experimental side but two animals used LFRs consistently. The reappearance of LFRs suggested that in some cases recruitment of alternative pathways occurs. This was tested in a second series of experiments.

Fig. 1 Reconnected nerve trunks 49 h after lesion. Large nerve trunk running through the section had been cut at a branch point. Arrows are arranged on either side of the healed scar. Note apparent disorganisation within nerves across the scar. Bar, 200 μm.

Ten animals were set out as before and tested for turning, which they all did. A cut was made across one side and they were tested for turning 19 h later. The experimental sides used LFR 94% of the time. Two of these animals did turn, one in two out of thirteen tests and the other in four of eleven tests. Table 2 shows that when the cut had rehealed 46 h later all the animals turned to the experimental side with only occasional LFRs. Once these readings were taken the original cuts were reopened and the animals retested 16 h later. Following the initial cut turning had only been recorded 5.6% of the time before healing but when the cut was reopened turning was recorded 72.1% of the time. The difference between these two results is statistically significant ($P < 0.001, \chi^2$ test). All of the animals turned but seven also gave occasional LFRs. Turning behaviour to the reopened cut sides differed from that recorded after the cuts had healed. There was a considerable latency between contact and the initiation of turning which could last as long as 20 s in the reopened animals, whereas it was usually only 1–5 s in healed animals. The fact that animals still turned following reopening of the cut suggests that mechanical coupling is not a factor in turning elicitation. Histological examination of the reopened lesions indicated that in all but one case the nerves on the healed side had been completely resevered.

These results suggest quite strongly that functional connections can be made between severed portions of the nerve trunks and also that alternative, perhaps pre-existing, pathways can be recruited as well. Although such systems may have been suspected in these primitive systems previously, this seems to be the first time that such recovery of function has been clearly demonstrated.

HAROLD KOOPOWITZ
DAVID SILVER
GREG ROSE

Department of Developmental and Cell Biology,
University of California
Irvine, California 92664

1 Olmstead, J. M. D., *J. exp. Zool.*, **36**, 49–56 (1922).
2 Best, J. B., and Rubinstein, I., *Science*, **135**, 916–918 (1962).

Influence of haemoglobin type on the induced ovulation rate in sheep

HARRIS and Warren[1] first reported haemoglobin (Hb) polymorphism in sheep; they separated a fast moving Hb A, and a slower moving Hb B, which were controlled by a single pair of alleles yielding three phenotypes—AA, BB and AB (ref 2). Several investigators have subsequently reported on the influence of Hb type on the reproductive performance of sheep. Evans and Turner[3] found that Merino ewes of Hb types BB and AB were more fertile and had a greater proportion of multiple births than AA ewes; subsequent investigations have confirmed this observation[4]. In a study of Australian Merino ewes grazing oestrogenic clover pastures Obst *et al.*[5] reported that AA and BB ewes were significantly more fertile than BB animals, suggesting that the A gene is associated with some factor which protects against the deleterious effects of plant oestrogens. We report observations in Indian Bikaneri ewes and suggest an association between Hb type and the ovarian response to exogenous gonadotrophins.

Seventy-eight adult multiparous Bikaneri ewes were classified according to their Hb phenotypes to give 12 AA, 36 BB and 30 AB animals. They were all kept in identical conditions of housing and management, and oestrus was detected by raddled vasectomised rams at 0900 daily; the day of onset of oestrus was classified as day 0. The BB and AB animals were subdivided into four groups and were injected with either 0, 500, 750 or 1,000 IU of pregnant mare serum gonadotrophin (PMSG,

Table 1 Influence of Hb type on ovarian response to PMSG in Bikaneri ewes

			Mean number of ovulations ($\pm$s.e.)				
PMSG level (IU)		0		500		750	1,000
Hb Type	n		n		n		n
AA	4	1.0		—		—	8 1.0 (nil)
BB	6	1.0	10	1.6$\pm$0.33 (3)	12	2.08$\pm$0 077 (11)	8 3.5$\pm$0.717 (7)
AB	6	1.0	8	1.25$\pm$0.070 (2)	8	3.0$\pm$1.20 (6)	8 5.25$\pm$2.20 (8)

n, Number of observations

Figures in parentheses indicate the number of animals which responded to PMSG treatment. Only those animals which had two or more corpora lutea in the ovaries were considered to have responded.

Gestyl, Organon) intramuscularly on the 13th day after oestrus. There were insufficient AA ewes to form more than two groups, which were either untreated or given 1,000 IU PMSG. The number of ovulations in response to this gonadotrophin treatment was assessed by counting the corpora lutea at laparotomy on the 5th to 7th day of the cycle following PMSG injection (Table 1).

None of the AA animals responded to the higher dose of PMSG, whereas there was a significant response ($P < 0.01$) of the AB and BB animals which was dose dependent (χ^2 test). The variation in the response of AA, BB and AB animals to 1,000 IU PMSG treatment was significant ($P < 0.01$) while between BB and AB animals was not significant (χ^2 test).

The ovulation rate of mammals is thought to depend primarily on the amount of gonadotrophins released into the blood[6] rather than in changes in ovarian sensitivity to gonadotrophins[7]. The results of the present study strongly suggest that the number of follicles which will ovulate may also be determined by ovarian sensitivity. In analogous studies in mice, genetic differences in ovulation rate have been attributed to differences in ovarian sensitivity[8-10].

Bindon et al.[11] reported that in a flock of Merino ewes selected for fecundity the ovarian response to a standard dose of PMSG was significantly higher in the group with the higher incidence of multiple births. Since response to PMSG is partly dependent on the endogenous gonadotrophin concentrations[12,13], a higher concentration of endogenous gonadotrophin in the BB or AB ewes may be responsible for the higher ovulation rate to PMSG. This possibility seems to be remote, however, since, irrespective of the Hb type, the incidence of twinning in the Bikaneri ewes is exceptional (2–3%). It may well be that the rate of secretion of endogenous gonadotrophin in BB and AB ewes may be lower than in AA ewes. Recent studies have revealed a significant variation in the peripheral plasma LH concentrations between ewes of same breed but of two different strains[14]. It also remains to be determined whether the sensitivity of BB and AB ewes to other hormones is also higher than that of AA ewes.

The results of this study suggest that haemoglobin type may provide a simple means of selecting sheep for increased sensitivity to gonadotrophins, and may also explain some of the variability in response to standard doses of gonadotrophin encountered in practice[15,16].

This work was supported by a grant from the Indian Council of Agricultural Research. We thank Dr R. V. Short for reading the manuscript and for suggestions.

H. C. PANT
M. D. PANDEY
Department of Physiology and Biochemistry,
Veterinary College, PO Mathura (U.P.), India

1 Harris, H., and Warren, F. L., *Biochem. J.*, 60, 29 (1955)
2 Evans, J. V., King, J. W. B., Cohen, B. L., Harris, H., and Warren, F. L., *Nature*, 178, 849 (1956).
3 Evans, J. V., and Turner, H N , *Nature*, 207, 1396 (1965).
4 Seth, O. N , *Curr Sci.*, 37, 231 (1968)
5 Obst, J. M., Seamark, R. F., and McGowan, C J , *Nature*, 232, 497 (1971).
6 Fowler, R E., and Edwards, R. G., *J. Endocr.*, 15, 374 (1957).
7 Nalbandov, N. V., in *Reproductive Physiology*, second ed., 76 (Freeman, W. H., San Francisco.
8 McLaren, A , *J Endocr.*, 25, 137 (1962).
9 Land, R. B., and Falconer, D. S , *Genet. Res* , 13, 25 (1969).
10 Bindon, B. M., and Pennycuik, P. R., *J. Reprod Fert.*, 36, 221 (1974)
11 Bindon, B M., Chang, T. S., and Turner, H. N., *Aust. J. Agric. Res.*, 22, 809 (1971).
12 Lamond, D. R., and Emmens, C. W., *J. Endocr* , 18, 251 (1959).
13 Lamond, D. R., and Bindon, B. M., *Nature*, 209, 512 (1966).
14 Thimonier, J., and Pelletier, J., *Annls Biol anim. Biochim. Biophys* , 11, 559 (1971).
15 Wallace, L. R., *J agric. Sci., Camb* , 45, 60 (1955)
16 Gordon, I., *J. agric. Sci , Camb.*, 50, 123 (1958)

Implication of absence of HCG-like gonadotrophin in the blastocyst for control of corpus luteum function in pregnant rabbit

IN the primate, the corpus luteum formed after ovulation has a relatively short life span. If the ovum becomes fertilised, however, the presence of the embryo in the uterus leads to 'rescue' of the corpus luteum and a prolongation of its functional life[1]. Circumstantial evidence suggests that this is achieved through the elaboration of a luteotrophic substance (chorionic gonadotrophin) by the trophoblastic cells of the implanting embryo[1,2]. Based on differences in the plasma progesterone levels of pregnant and pseudopregnant animals, a similar situation has been proposed for the rabbit[3]. In support of this, the blastocyst fluid from 6-d pregnant rabbits has been reported to contain a substance similar to human chorionic gonadotrophin (HCG), by a radioreceptor assay[4], and a substance similar to luteinising hormone (LH), by a heterologous radioimmunoassay[5]. Our study was initiated to identify definitively the source of the HCG-like substance in the rabbit blastocyst. Using a highly sensitive *in vitro* bioassay method we have examined the concentration of HCG-like material in the serum throughout pregnancy and in the blastocyst from days 4 to 7 of pregnancy. We report here that the rabbit blastocyst does not contain or secrete any gonadotrophin which has significant HCG-like activity.

New Zealand White rabbits were mated and killed 4–6 d later. The uterine horns were removed and flushed from the oviducal end with physiological saline to expel the embryos, which were immediately transferred to incubation vials. The surrounding saline was aspirated and appropriate materials added for incubation. To obtain blastocyst fluid without loss of any critical material, the following procedure was adopted. Uterine horns removed on days 6 and 7 of pregnancy were wrapped in aluminium foil and frozen rapidly by immersing in an acetone–dry ice mixture. The uteri were then partially thawed, dissected longitudinally and the frozen blastocysts transferred to centrifuge tubes. After thawing, the blastocysts were ruptured, centrifuged and the supernatant obtained. The
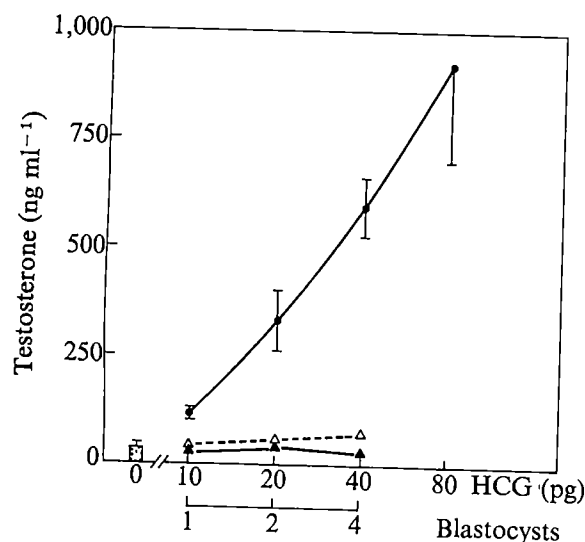
Fig. 1 Testosterone production by isolated mouse testes. Mice (70–80-d-old) were killed by cervical dislocation and the testes removed. After decapsulation the testes were rinsed with cold saline and placed in 16×50 mm glass vials containing 1 ml incubation medium (Krebs–Ringer bicarbonate buffer, pH 7.4, with 0.1% glucose) and graded doses of HCG or samples. HCG (10,600 IU mg⁻¹) was added in 100 μl incubation medium with 0.1% bovine serum albumin (●). Using saline, blastocysts were flushed out from the uterine horns of 6-d-pregnant rabbits and immediately transferred to incubation vials. The blastocysts were either left intact (▲) or punctured (△) with a needle and the blastocoel fluid allowed to escape. All points were assayed in quadruplicate. Incubations were carried out at 34 °C in a metabolic shaker at 150 cycles min⁻¹ in an atmosphere of 95% O₂–5% CO₂. After 4 h incubation aliquots of incubation medium were diluted and stored at −20 °C until the determination of testosterone by a direct radioimmunoassay⁶.

endometrial surface was scraped with a blade and the material coating the endometrium as well as part of the endometrium obtained. This was weighed, homogenised with 10 volumes saline, centrifuged and the supernatant obtained. The *in vitro* bioassay for HCG- and LH-like substances was based on the steroidogenic response of the mouse testes⁶,⁷.

Figure 1 shows the testosterone production by decapsulated mouse testes. A dramatic increase in testosterone production was obtained with 20 pg of HCG (biological activity 10,600 IU mg⁻¹). When testes were incubated along with day 6 blastocysts, up to four freshly-obtained blastocysts did not stimulate testosterone production. This was the case for intact blastocysts as well as for blastocysts which were disrupted and the blastocoel fluid allowed to escape into the medium.

The steroidogenic response of a highly sensitive mouse Leydig cell preparation was used to evaluate the HCG-like activity (Fig. 2). Pregnant rabbit serum obtained 2 h after mating gave a dose–response curve parallel to that of HCG. In this system 200 μl day 7 blastocyst fluid or 100 μl of day 6 blastocyst fluid did not show any HCG-like activity. Saline extracts from 10. mg endometrial scrapings from 6- and 7-d-pregnant rabbits also showed no activity. To ascertain whether blastocysts secrete HCG-like material, embryos were flushed out of the uterus on day 4 of pregnancy and preincubated for 21 h. Leydig cells were then added and incubation continued for a further 3 h. No evidence for the secretion of HCG-like material was noted.

The Leydig cell preparation was used to measure HCG-like activity in the rabbit serum throughout pregnancy. By 0.5 h after coitus there was a 100-fold increase in the HCG-like activity (Fig. 3). After reaching maximum levels at 2 h *post coitum* the activity declined by 4 h and had returned to premating levels by 24 h. The levels remained low throughout pregnancy and did not rise above the premating levels. The recovery of HCG added to sera or blastocysts was quantitative.

These results demonstrate that coitus is followed by a rapid increase in the serum HCG-like activity which reaches a peak in 1 to 2 h and then decreases quickly to premating levels, at which it remains throughout pregnancy. The post-coital LH levels measured by radioimmunoassay show a similar pattern⁸. No significant HCG-like activity could be detected in the blastocysts between days 4 and 7 of pregnancy. Haour and Saxena⁴, using a radioreceptor assay, reported that on day 6 of pregnancy the concentration of HCG-like material in the blastocoel fluid was tenfold higher (87 ng ml⁻¹) than that in the serum (6–8 ng ml⁻¹). They also reported that the plasma levels of HCG-like material in the 6-d-pregnant rabbit was significantly higher than that in the non-pregnant animal. Results of the present study which are based on a highly sensitive *in vitro* bioassay do not confirm the results obtained by radioreceptor assay. This discrepancy is probably related to the different methods used. Comparison of the radioreceptor assay with radioimmunoassay and *in vitro* bioassay has shown that the radioreceptor assay was subject to nonspecific interference by plasma proteins⁹.

In the rabbit, a reflex ovulator, mating induces ovulation and corpus luteum formation. If the ova are not fertilised a pseudopregnancy of 17 d duration follows. The corpus luteum of pseudopregnancy shows regressive changes by day 12. Most of the evidence suggests that the non-pregnant rabbit uterus produces a luteolytic substance, presumably a prostaglandin, which limits the duration of corpus luteum function¹⁰. The biological evidence for the existence of a luteotrophic substance derived from the conceptus before day 10 of pregnancy is equivocal. Using a competitive protein-binding assay plasma progesterone levels were reported to be significantly higher in pregnant rabbits during the preimplantation stages than in pseudopregnant animals at similar stages³. A radioimmunoassay, however, showed no such differences¹¹.

The significance of the detection of HCG-like substance by indirect immunofluorescence on the surface of mouse embryos
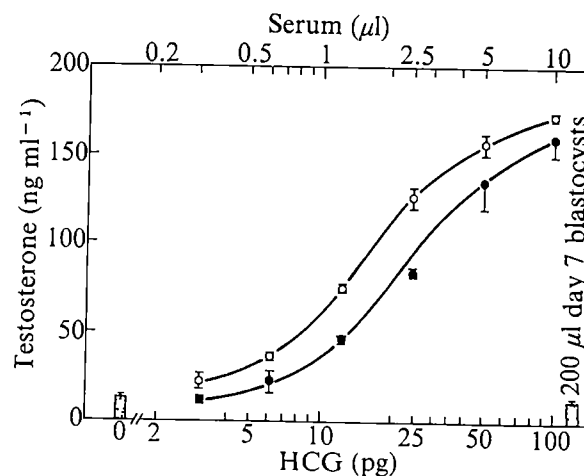

Fig. 2 Testosterone production by mouse Leydig cell preparation. Four mice were killed by cervical dislocation and the testes removed. After decapsulation they were rinsed with saline and placed in a beaker with 25 ml medium 199 (Difco) containing 2% foetal bovine serum. Testes were chopped into small pieces with scissors and stirred for 10 min at room temperature on a magnetic stirrer. The medium was then filtered through a fine nylon mesh and centrifuged at 600g for 15 min at 4 °C. The supernatant was discarded and the cells resuspended in 25 ml medium. A portion (0.25 ml) of the cell suspension was added to tubes containing 0.1 ml standards or samples. HCG (●) was dissolved in the incubation medium. Serum obtained 2 h *post coitum* (○) from two rabbits was pooled and diluted with incubation medium. Blastocyst fluid (vertical bar) from 7-d-pregnant rabbit was lyophilised and reconstituted in the incubation medium to half its original volume. The rest of the procedure was as in Fig. 1 except that the incubation time was reduced to 3 h; stippled column, control.
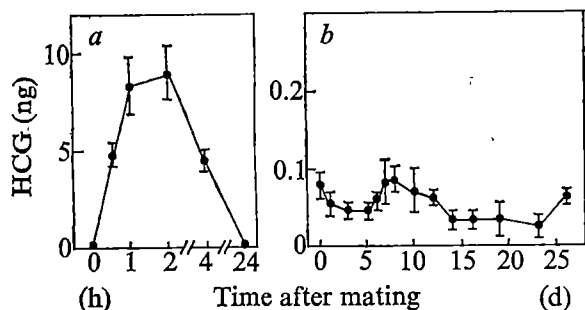
Fig. 3 Serum levels of HCG-like material in the pregnant rabbit measured as in Fig. 2. Blood samples (3–4 ml) from the marginal ear vein were collected before mating, at frequent intervals after mating and at less frequent intervals throughout pregnancy. The high HCG activity at 1 and 2 h *post coitum* (*a*) represents the presumptive LH peak. The levels are much lower throughout pregnancy (*b*, note change in scale).

during the morula stage but not during the blastocyst stage is not clear[12]. A role for HCG in the protection of the foetus against attack by the maternal immunological system has been suggested[13]. Perhaps substances resembling HCG reported to be present in the unimplanted mouse and rabbit embryos play a role in the immunological system but are not gonadotrophins.

We thank the NIH for HCG (CR117) and for financial support.

KALYAN SUNDARAM
KATHRYN G. CONNELL
THOMAS PASSANTINO

The Population Council,
The Rockefeller University,
New York, New York 10021

1 Knobil, E., *Biol. Reprod.*, **8**, 246–258 (1973).
2 Tullner, W. W , *Acta Endocr (Kbh)*, suppl., **166**, 200–213 (1971).
3 Fuchs, A. R., and Beling, C. C., *Endocrinology*, **94**, 1054–1058 (1974).
4 Haour, F., and Saxena, B. B., *Science*, **185**, 444–445 (1974).
5 Fujimoto, S., Euker, J S., Riegel, G. D., and Dukelow, W. R., *Proc. Japan Acad.*, **51**, 123–125 (1975).
6 Dufau, M. L., Catt, K J., and Tsuruhara, T., *Endocrinology*, **90**, 1032–1040 (1972).
7 VanDamme, M. P., Robertson, D. M., and Diczfalusy, E., *Acta Endocr (Kbh)*, **77**, 655–671 (1974).
8 Scaramuzzi, R. J., Blake; C A., Papkoff, H., Hilliard, J., and Sawyer, C. H , *Endocrinology*, **90**, 1285–1291 (1972).
9 Catt, K. J., Dufau, M. L., and Vaitukaitis, J. L., *J. clin. Endocr. Metab.*, **40**, 537–540 (1975).
10 Hilliard, J., *Biol. Reprod.*, **8**, 203–221 (1973).
11 Holt, J. A., Wilson, S M., and Keyes, P. L., *Fedn Proc.*, **34**, 592 (1975).
12 Wiley, L. D., *Nature*, **252**, 715–716 (1974).
13 Martin, B. J., Spicer, S. S., and Smythe, N. M., *Anat. Rec.*, **178**, 769–786 (1974).
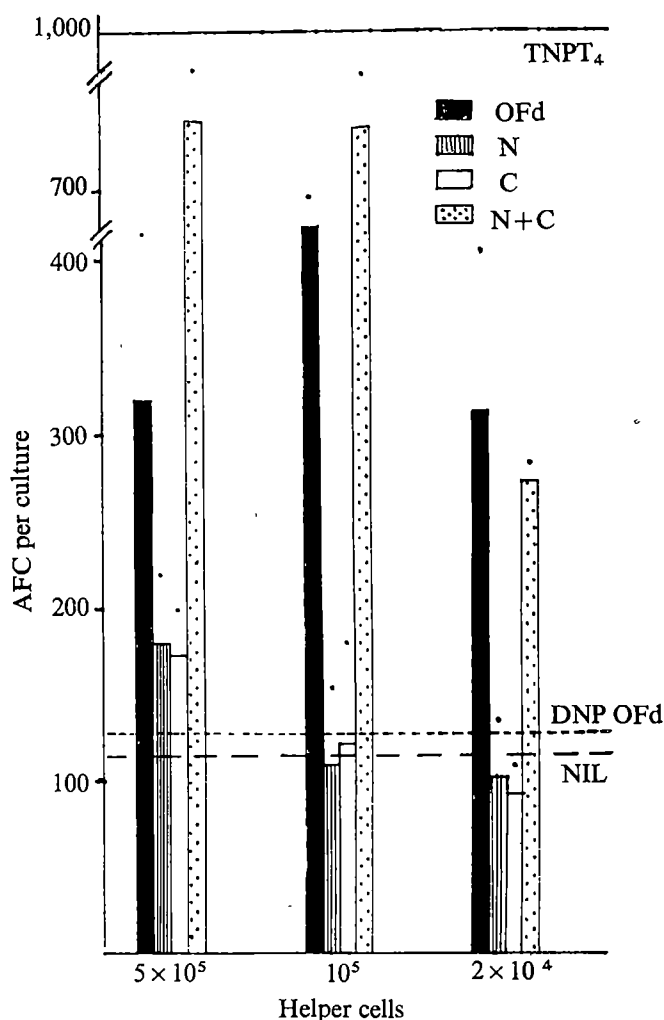
Fig. 1 Synergy between N and C primed T cells, Spleen cells were treated with a cytotoxic rabbit anti-mouse B cell antiserum, prepared by repeated injection (3 or 4) of anti-θ treated spleen cells which had been depleted of dead cells[22] and red cells[23]. About 10[8] cells were injected at 2-weekly intervals, half intramuscularly emulsified in Freund's, and the rest intravenously. B cell contamination after treatment with the antiserum and complement ranged from 0–3% in replicates. Significant helper cells to OFd were only induced provided OFd primed T cells, or a mixture of N and C-BSA primed spleen cells were used. The dots above the bars indicate the upper limit of the standard error.

T–T interaction in the generation of helper cells *in vitro*

THE interaction of different cell types is now widely recognised as being of fundamental importance in the generation of many different immune responses[1,2], and occurs in many species. Several broad groups of interactions have been described—between macrophages and lymphocytes[3,4], between T cells, B cells (and macrophages) in antibody production[1,3,5] and between subpopulations of T cells in the graft versus host responses[6], and in the generation of cytotoxic T cells[7-10].

Levy and her colleagues have reported that the antigen performic acid oxidised ferrodoxin (OFd), derived from *Clostridium pasteurianum* has only 2 major antigenic determinants located in the carboxyl and amino terminal regions of the molecule[11]. They reported that cooperation between cells primed to the determinants situated at the COOH- (C-hapten) and at the NH_2-terminus (N-hapten) is necessary for the generation of a proliferative response of guinea pig lymph node cells *in vitro* to the whole antigen, OFd [12,13], but not for the expression of hypersensitivity reactions. Having recently described a system for the development of helper cells *in vitro*[14], we wanted to

determine whether the T helper cell response to OFd required cooperation between two populations of T cells, in analogy with other cooperative proliferative response to OFd[11].

By using the chemically defined determinants of OFd either linked together as in the whole antigen, or separately, coupled to bovine serum albumin (BSA), it was possible to prime cell populations *in vivo* to both or to either of the individual determinants and subsequently to use these cells *in vitro* to test for cell interaction during the development of helper cells to OFd. Cells were initially cultured for 4 d in the presence of OFd to generate helper cells directed towards OFd, and then small numbers of these cells were transferred to a second culture where, in the presence of excess B cells and macrophages and with dinitrophenylated-OFd (DNP-OFd) helper cells directed towards OFd would augment the plaque forming cell response to DNP. Here we describe the use of three distinct experimental approaches which have previously been used to study T–B interactions. They demonstrated that the interaction of two T-cell subpopulations is essential for the development of helper cells to OFd *in vitro*.

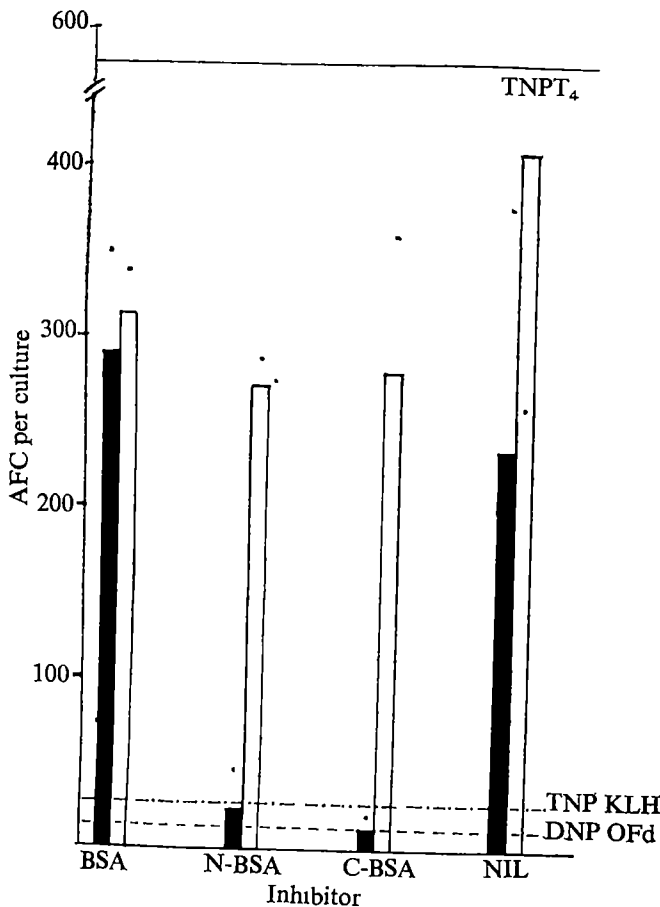The first approach was the classical one of synergy, which

Fig. 2 Effect of excess N or C hapten on the response to OFd. Either bovine serum albumin (BSA) or N-BSA or C-BSA was added to cultures of OFd primed T cells containing OFd (1 μg ml⁻¹) and KLH (0.1 μg ml⁻¹) N-BSA, C-BSA or N or C-S-BSA (made with succinylated BSA) and N or C poly-D-glutamic acid (PDG) all suppressed the helper response to OFd. Substitution ratios of the preparations used ranged from 10–20 N or C groups mol⁻¹. These were made as described previously[12]. All these compounds were toxic in high concentrations, with nonspecific diminution of the KLH response beginning at 30 μg ml⁻¹ of N or C-PDG, and 100 μg ml⁻¹ of N or C-BSA, or succinylated BSA. The inhibition results shown were with 10 μg ml⁻¹ of N or C-BSA. Inhibition was also found with 1 μg ml⁻¹ of N or C-BSA, but not with 0.1 μg ml⁻¹. Dot above the bar graph indicates the standard error. Dark bar represents the response to DNP OFd, the unshaded bar represents the response of the same cultures to TNP KLH.

implies that a mixture of two cell populations yields a response substantially greater than the sum of the two individual populations. Over the past year, four series of mice have been used for these experiments, each series comprising three groups of mice, which were primed with either the N or the C hapten conjugated to BSA (that is N-BSA), or with OFd. Spleen cells from these mice or mixtures of cells primed to N-BSA or C-BSA were cultured with OFd. Typical results are shown in Table 1 and Fig. 1. Experiments 1 and 2 of Table 1 show that cells primed to N-BSA or C-BSA do not yield significant numbers of helper cells. Mixtures (1:1) of these cells, however, produced responses as good as OFd primed cells and sometimes significantly better (Fig. 1). Since neither N nor C primed cells yield measureable help, it is not possible to calculate the exact degree of synergy, but this can be estimated to be at least 25-fold, and often 500-fold (Table 1). That the interacting N and C primed cells were T cells is shown by experiment 2 of Table 1 and Fig. 1, which indicates that nylon wool filtered spleen cells (<10% B), or anti-B and complement-treated spleen cells (<3% B) cooperated even more efficiently with B cells than helper cells obtained from unpurified spleen (experiment 1). Experiments

with anti-θ antisera verified that the helper cells were, as usual, T cells (data not shown).

Inhibition of cooperative responses by unconjugated carrier or hapten has been reported both *in vitro*[16] and *in vivo*[15]. Responses that do not involve cell cooperation are not blocked in this way[16]. Thus, the effect of adding N-BSA or C-BSA on the response of OFd primed T cells to OFd was ascertained. Figure 2 shows that the addition of 10 μg ml⁻¹ of N-BSA or C-BSA suppressed the response to 1 μg ml⁻¹ OFd. Significant but not complete suppression was obtained with 1 μg ml⁻¹ N-BSA or C-BSA (data not shown). Analogous results were obtained with N or C hapten coupled to poly-D-glutamic acid, or to succinylated BSA (data not shown). As control, the helper cell response to KLH was also assayed with the same pool of helper cells, to exclude the possibility that the suppression of the OFd response in the presence of N or C-BSA was due to nonspecific toxicity.
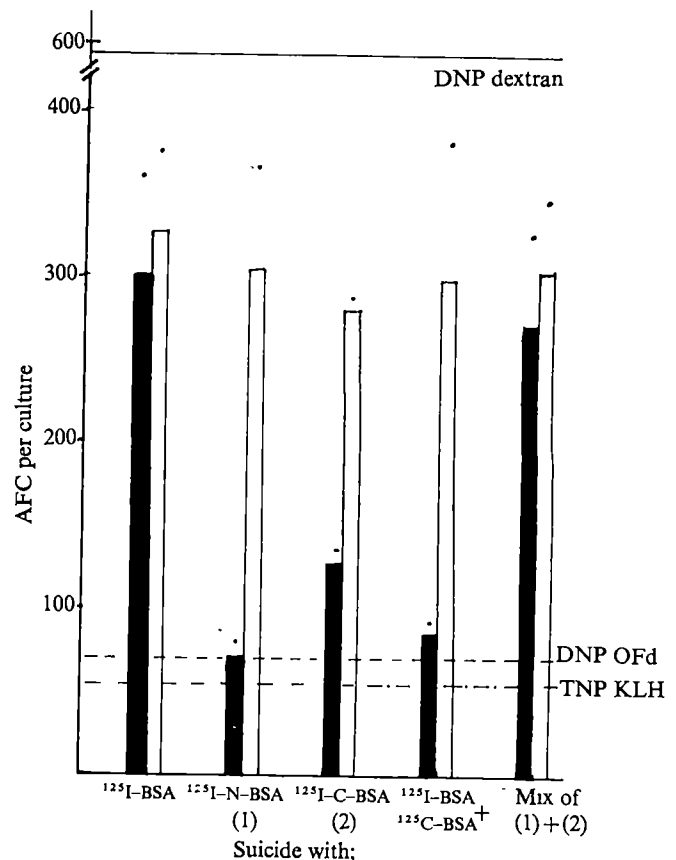

Fig. 3 Antigen induced suicide with radiolabelled N or C-BSA. This was performed in a way similar to that of Byrt and Ada[24] and Pearson *et al.*[13]—BSA, N or C-BSA were radiolabelled with ¹²⁵I using the chloramine-T method, 2–10 μg of each compound was labelled with 1 mCi of carrier-free ¹²⁵I-iodide (Radiochemical Centre, Amersham). Primed T cells (10⁸) (purified by nylon wool) were incubated with 1 μg ¹²⁵I-labelled antigen for 1 h at 0 °C in HEPES-buffered Eagle's medium (sometimes containing azide) then washed three times through foetal calf serum, and left overnight at 4 °C before culture with OFd and KLH, together with the addition of 5×10⁵ anti-T and anti-B serum treated peritoneal macrophages per culture. Dark bar represents response to DNP OFd. Unshaded bar represents the response to TNP KLH. Dot above bar graph indicates the upper limit of one standard error of the mean. Results shown are with 10⁵ helper cells from suicided or mixed back helper cell cultures, which were subsequently cultured with normal spleen cells and DNP OFd or TNP KLH. Specific suicide of the response occurred with ¹²⁵I-N-BSA or ¹²⁵I-C-BSA. It is interesting that mixing the two suicided populations completely restored the response to OFd. These experiments were performed as a helper cell dose-response titration, using 5×10⁵, 10⁵ and 2×10⁴ helper cells. For simplicity, the results with 5×10⁵ and 2×10⁴ are not included, as they corroborated those shown.

Table 1 Synergy between N- and C-primed T cells in the generation of helper cells to OFd

Cells cultured	Helper cell induction			Cell cooperation	
	Treatment	Antigen	Helper cells transferred	Antigen	Anti-DNP response (AFC per culture)
(1) OFd primed spleen	nil	OFd	10^5	DNP OFd	173± 22
N-primed spleen	nil	OFd	10^5	DNP OFd	13± 10
C-primed spleen	nil	OFd	10^5	DNP OFd	0
N-+ C-primed spleen (1 : 1) —	nil —	OFd —	10^5 —	DNP OFd	217± 33
				DNP OFd	3± 43
				DNPPOL	553±160
				nil	27± 33
(2) OFd primed spleen	Nylon wool	OFd	5×10^5	DNP OFd	320±122
			10^5	DNP OFd	417± 65
	Nylon wool		2×10^4	DNP OFd	313±100
N-primed spleen	Nylon wool	OFd	5×10^5	DNP OFd	187± 53
			10^5	DNP OFd	110± 47
C-primed spleen	Nylon wool	OFd	5×10^5	DNP OFd	173± 30
			10^5	DNP OFd	123± 62
N-primed + C-primed (1 : 1)	Nylon wool	OFd	5×10^5	DNP OFd	743±105
			10^5		717± 82
			2×10^4	DNP OFd	267± 17
—	—	—	—	DNP OFd	127± 25
				TNP T$_4$	920±110
				nil	113± 54

CBA mice bred at University College or at the ICRF Animal Unit at Mill Hill were used at 70–210 d of age. For use as donors of primed spleen cells, they were injected intraperitoneally with 25 μg of OFd, N- or C-BSA emulsified in Freund's complete adjuvant. Usually the N or C hapten was conjugated to succinylated BSA. 15×10^6 spleen cells were cultured with 1 μg ml^{-1} OFd for 4 d to yield helper cells. Cell recoveries were variable from 10–45%, but averaged 30%. Washed viable (Trypan blue exclusion) cells from these cultures were mixed in the numbers indicated with 15×10^6 unprimed CBA spleen cells and 1 μg ml^{-1} DNP OFd and cultured for a further period of 4 d before assay for anti-DNP antibody forming cells using the Cunningham technique[14]. Preliminary experiments had indicated that the two periods of 4 d and 1 μg ml^{-1} of antigen were optimal. Eleven experiments of the type shown as experiment 1 have been performed. No helpers were obtained with N or C primed cells at other times of culture (1–6 d), or other concentrations of OFd.

In experiment 2, spleen cells from OFd, N or C primed mice were passed through nylon wool columns[20] to purify T cells. Contamination with B cells, as assessed by fluoresceinated rabbit anti-mouse Ig antibody was 5–9% in replicate experiments. Because the nylon wool columns also remove macrophages, essential for helper cell induction[21] 5×10^6 peritoneal exudate macrophages (obtained 3 d after the injection of starch), purified by treatment with anti-B serum and complement[21] were added to each helper cell culture. The exact degree of synergy is difficult to estimate, as neither N nor C primed cells yield detectable help A crude approximation of the degree of help may be obtained by comparing the smallest numbers of (N and C) mixed cells which provide help, for example, 2×10^4, with those unmixed that do not, for example 5×10^5, giving a synergy ratio of greater than or equal to 25. DNPPOL (dinitrophenylated polymeric flagellin) and TNPT$_4$ (trinitrophenylated phage T$_4$) are thymus-independent antigens

The technique of radioactive antigen-induced suicide was also used to verify the requirement for both N-hapten and C-hapten reactive cells to generate helper cells to OFd. Incubation of OFd primed spleen cell or purified T cells with highly radioactive (^{125}I-labelled) N or C-BSA suppressed the helper response to OFd. In contrast, the responses of the same cell pool to KLH was unimpaired. ^{125}I-BSA was not suppressive. Mixing the N-suicided and the C-suicided populations (each of which should contain an intact population of cells directed against the other hapten) restored the OFd helper response to normal levels, thus providing further evidence for a cooperative interaction between the two hapten reactive cells.

Three independent experimental approaches have thus all indicated that N and C reactive T cells interact to generate helper cells to OFd. It seems then that there is T–T interaction in the development of helper cells, just as in other T cell responses—the graft versus host response[6] and the T killer response[7–10]. Other studies also support our evidence for T–T interaction in the generation of helper cells. Either anti-lymphocyte serum (ALS) treatment or adult thymectomy (ATX) markedly reduces the thymus-dependent antibody response to tobacco mosaic virus[17]. Furthermore, Hunter, Kappler and colleagues have also shown that both ALS and ATX diminish helper responses to sheep red cells, but that both procedures are needed to abolish the helper response[18,19]. Our studies *in vitro*, using keyhole limpet haemocyanin as antigen, indicated that defined populations of T$_1$ and T$_2$ cells purified from the spleen (by treatment with ALS and by adult thymectomy respectively) do not yield helper cells unless mixed together (unpublished data), in contrast to normal spleen which contains both T$_1$ and T$_2$ cells. Thus the interaction of subpopulations of T cells is of importance in the generation of helper cells, just as it is in the development of graft versus host and the cytotoxic response.

We thank Mrs M. Dunkley, Miss E. Creaghe and B. de Souza for assistance.

MARC FELDMANN

ICRF Tumour Immunology Unit,
Department of Zoology,
University College London,
Gower Street,
London WC1E 6BT, UK

D. G. KILBURN
J. LEVY

Department of Microbiology,
University of British Columbia,
2075 Westbrook Place, Vancouver,
British Columbia, V6T 1W5 Canada

1 Miller, J F A P, Basten, A., Sprent, J., and Cheers, C., *Cell Immun*, 2, 469–495 (1971).
2 Feldmann, M, *Ser Hematologica*, 7, 593–609 (1974).
3 Feldmann, M, and Nossal, G. J V, *Transplant. Rev*, 13, 322 (1972)
4 Unanue, E. R, *Adv Immun*, 15, 95–135 (1972)
5 Katz, D H, and Benacerraf, B., *Adv Immun*, 15, 1–94 (1972)
6 Cantor, H, and Asofsky, R, *J. exp. Med*, 131, 235–262 (1970)
7 Wagner, H, *J exp Med*, 138, 1379–1397 (1973)
8 Tigelaar, R., and Feldmann, M, *Trans Proc*, 5, 1711)–1717 (1973).
9 Simpson, E, and Cantor, H, *Eur J Immun*, 5, 330–336 (1975).
10 Hayry, P, and Andersson, L C., *Eur J Immun.*, 4, 145–147 (1974).
11 Kelly, B, and Levy, J. G., *Biochem J.*, 10, 1763–1766 (1971).
12 Waterfield, D, Levy, J G, Kilburn, D G, and Teather, R M., *Cell Immun*, 3, 253–263 (1972)
13 Pearson, T., Levy, J G, and Kilburn, D. G., *Eur. J Immun.* (in the press).
14 Kontiainen, S., and Feldmann, M., *Nature new Biol.*, 245, 285–286 (1973)
15 Mitchison, N A, *Eur. J. Immun.*, 1, 18–27 (1971).
16 Feldmann, M, *Eur. J. Immun*, 2, 130–137 (1972)
17 Strobel, G., *Eur. J Immun*, 2, 475–476 (1972).
18 Kappler, J W., Hunter, P. C., Jacob, D, and Lord, E., *J Immun*, 113, 27–39 (1974)
19 Areano, B., Marrack (Hunter), P. C, and Kappler, J. W, *J Immun*, 114, 747–751 (1975).
20 Julius, M, Simpson, E, and Herzenberg, L. A, *Eur J Immun.*, 3, 645–649 (1973).
21 Erb, P., and Feldmann, M., *Nature*, 254, 352–354 (1975).
22 Von Bohmer, H., and Shortman, K, *J Immun. Methods*, 2, 293–301 (1973)
23 Boyle, W., *Transplantation*, 6, 761–768 (1968).
24 Byrt, P., and Ada, G. L., *Immunology*, 17, 503–516 (1969).

Concurrent inhibition by chlorpromazine of concanavalin A-induced lymphocyte aggregation and mitogenesis

A PLANT lectin from extract of Jack bean (concanavalin A) is known to initiate transformation *in vitro* of T lymphocytes into large, proliferating blast-like cells[1]. Knowledge of the mechanism whereby con A induces such changes is incomplete, but a necessary first step seems to involve the binding of con A to receptors on the surface membrane of the lymphocyte. Within minutes of binding of a mitogen, recognisable alterations in lymphocyte surface membrane physiology have been noted. These include increased phospholipid incorporation into the membrane[2], increased fluxes of ionic potassium[3] and calcium[4], and increased uptake of nucleosides[5], sugar[6], and amino acids[7]. In addition, con A has been reported to increase the fluidity of lymphocyte membranes after binding[8]. Internalisation of con A is not required for lymphocyte activation so that con A binding to the lymphocyte surface membrane of T cells seems to be sufficient to induce blastogenesis[9].

Less attention has been paid to the fact that con A also induces an aggregation or clustering of mononuclear cells following its addition to *in vitro* culture. At optimal mitogenic concentrations, this process is not an immediate agglutination but occurs progressively over several hours in culture. It has been suggested that such cluster formation may be a necessary prerequisite for blast transformation induced by a mitogen, as cells immobilised in single cell suspensions in agar will not transform[10], and increased cell density[11], presumably augmenting cell–cell interaction, enhances mitogenesis. In accordance with this hypothesis we have found that similar concentrations of the surface membrane-active drugs, chlorpromazine (CPZ) will inhibit both the blastogenic response as well as the clustering of mouse spleen cells by con A.

The effect of CPZ on con A-induced blastogenesis is shown in Fig. 1. Concentrations of CPZ > 0.005 mM totally inhibited the uptake of ³H-thymidine by con A-stimulated cultures. Concentrations of CPZ < 0.001 mM had little effect.

When dealing with pharmacological inhibition studies such as this it is imperative that any cytotoxic effects of the drugs themselves be evaluated. Mouse spleen cells were therefore incubated with inhibiting concentrations of CPZ for 24–48 h in the presence or absence of con A. No loss of viability was seen at concentrations ≤ 0.05 mM by Trypan blue exclusion or ⁵¹Cr release assays. In addition, if spleen cells were pre-incubated for 2 h in the presence of concentrations of the drug (0.01 mM) sufficient to inhibit mitogenesis, washed extensively, and then cultured with con A, there was no difference in the blastogenic response compared with untreated control cells (data not shown). Thus, CPZ in these concentrations was not detectably cytotoxic to lymphocytes and its effect was reversed by removal of the drug. To further prove viability and characterise the inhibition of blastogenesis, CPZ was added at intervals after incubation of spleen cells with con A. If CPZ was added with con A or 2 h later, marked depression of thymidine incorporation was observed, whereas if CPZ was added after 24 h a response very similar to cultures lacking CPZ was observed (Fig. 2). This experiment provides additional proof of viability, as ³H-thymidine incorporation in response to con A proceeded unaltered in the presence of the drug. In addition, the inhibitory effect of the drug seems not to derive from an inhibition of DNA synthesis, that is, once the con A-induced intracellular message had been sent and the lymphocyte was committed to respond, the addition of the drug had no effect on the subsequent blastogenic response.

Con A-treated lymphocyte cultures were observed by light microscopy. Aggregation was far advanced by 12 h in the

absence of CPZ. Con A-induced lymphocyte aggregation was inhibited by CPZ at concentrations equal to that which inhibits blastogenesis. CPZ (0.01 mM) added to con A cultures after 12 h, however, did not disperse already formed cell clusters.

One possible mechanism of both inhibition of blastogenesis and of cluster formation could be the inhibition by CPZ of binding of con A to the surface of the lymphocyte. To test this possibility, binding studies using ³H-labelled con A were carried out. A 1 : 5 dilution of ³H-con A (80–92 Ci mM⁻¹) was made and diluted in a solution of 100 µg ml⁻¹ unlabelled con A. This was added to a population of spleen cells (5 × 10⁵ cells ml⁻¹), incubated for 30 min at 37 °C, washed three times with 1% albumin solution in phosphate-buffered saline, collected as above, and counted in a scintillation counter. The
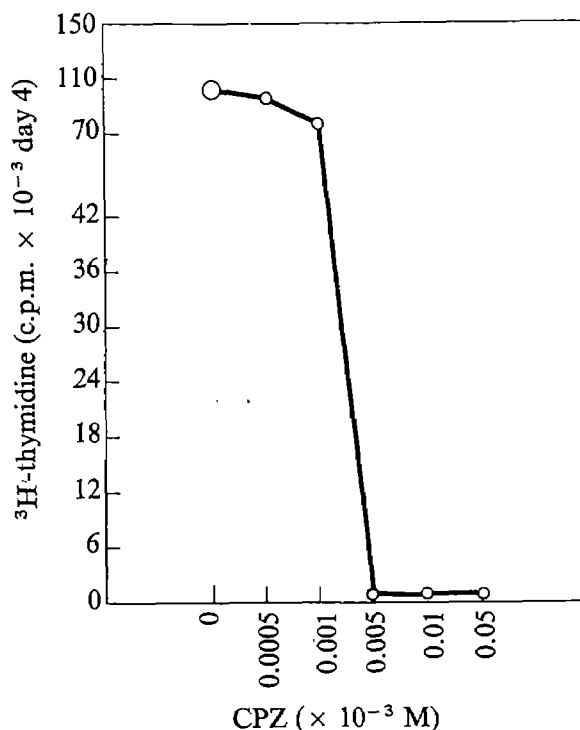
Fig. 1 CPZ dose–response inhibition of con A-induced ³H-thymidine incorporation of murine (C3H/HeJ) spleen cells: 5 × 10⁵ spleen cells were cultured in 0.2 ml medium 199 (GIBCO) containing 7.5% heat-inactivated human pooled serum and 1% penicillin and streptomycin. Cultures were carried out in triplicate in 96-well, flat bottom microtitre plates (Falcon). CPZ (Smith, Kline and French) was added in appropriate concentration immediately before addition of con A (5 µg ml⁻¹). At 18–24 h before collection, each well was pulsed with 1 µCi ³H-thymidine (6.7 Ci mM⁻¹). After 96 h culture, cells were collected in a 12-place mechanical collector (Otto Hiller), dried and counted in a Beckman LS330 scintillation counter. Each point represents the mean of triplicate cultures not differing by more than 10%.

concentrations of CPZ found to inhibit blast transformation had no effect on the binding of con A to the lymphocyte cell surface; however, α-methylmannose, an inhibitor of con A binding, did inhibit binding. As binding of ³H-con A is unaffected by CPZ, some event subsequent to lectin binding but necessary for both aggregation and blastogenesis must be at work. This is further supported by considerable data that CPZ acts primarily on the membranes of nerve, muscle and lymphocytes.

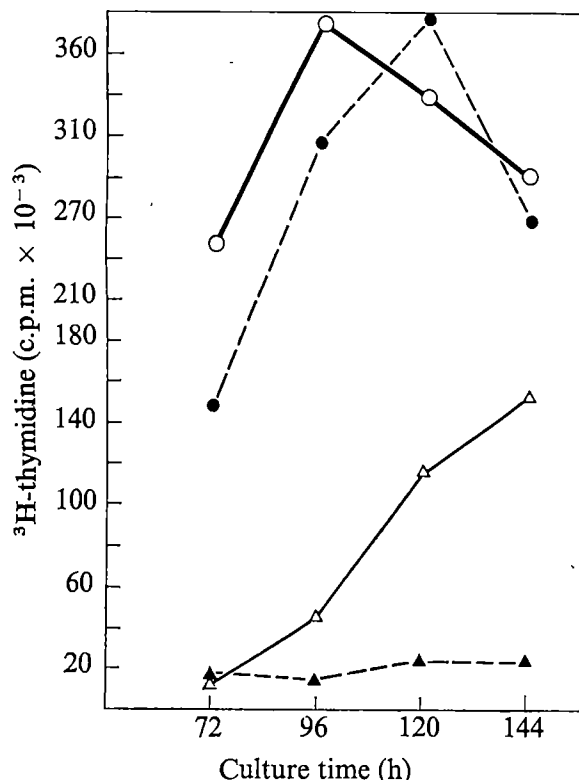Studies of lymphocyte activation have focused on the role of cyclic AMP[12] and cyclic GMP[13] as well as mitogen-induced

Fig. 2 Effect of timed addition of CPZ (0.01 mM) on con A-induced ^{3}H-thymidine incorporation. Cells were cultured and pulsed as in Fig. 1. Cultures were collected at times indicated on the abscissa. Con A (5 µg ml^{-1}) was added to all cultures (5×10^5 cells) at initiation of culture. CPZ (0.01 mM) was added at various times after culture. ○, No CPZ added; ▲, CPZ added with con A; △, CPZ added 2 h after con A; ●, CPZ added 24 h after con A.

changes in Ca^{2+} flux[14]. CPZ is a well established Ca^{2+}-blocking agent producing inhibition of Ca^{2+} fluxes in muscle[15] and adrenal preparations[16]. This pharmacological action of CPZ has been shown to be reversed by high Ca^{2+} concentrations in the media[17]. If, however, lymphocytes are cultured in media containing 5 or 10 mM calcium no reversal of CPZ inhibition of con A-induced blastogenesis was observed (data not shown).

CPZ has also been shown to inhibit hormonally stimulated increases in adenylate cyclase activity of rabbit heart tissue without affecting the base level of enzyme activity[18], and to decrease the amphetamine-induced increase of cyclic GMP of murine cerebellum[19]. Preliminary data indicate that addition of a range of concentrations of cyclic AMP, or its dibutyryl derivative (10^{-6}–10^{-3}M) did not reverse CPZ-induced inhibition of transformation or lymphocyte aggregation (data not shown). Cyclic GMP or its dibromo derivatives in a dose range of 10^{-9}–10^{-3} M either added to cultures with con A and CPZ or added to cells for 15 min before culture with con A and CPZ, also did not reverse the inhibitory effect of CPZ (data not shown). It seems, therefore that a membrane phenomenon not reversed by Ca^{2+}, cyclic AMP or cyclic GMP, is involved in the inhibition of con A-induced lymphocyte transformation by CPZ.

The cellular requirements for mitogen stimulation and their interactions in the activation process are possible targets for CPZ action. Highly purified human T cells show a markedly reduced response to mitogen-induced blastogenesis (J.R.S., and S. Hatfield, unpublished). This response, however, could be restored by the addition of isologous, allogeneic or even xenogeneic macrophages to mitogen cultures. This suggests that the macrophage plays a significant role in the response of T cells to mitogens. Additional support for the importance

of cellular interactions in lymphocyte activation by mitogens is given by Moorhead[11] who demonstrated an inverse and linear relationship between the surface area on which equal numbers of lymphocytes were cultured and the log of ^{3}H-thymidine incorporation into mitogen-stimulated cells. Kondrocki[10] has shown that isolated 'single-cell' suspensions of lymphocytes in agarose respond poorly to mitogen but that addition of erythrocytes or erythrocyte stromata to such preparations markedly increases the blastogenic response. Implicit in the above observations is that cell membrane interactions play an important role in mitogen activation. CPZ, as shown above, inhibits mitogen-induced lymphocyte clustering seen in con A-stimulated cultures and thus removes the opportunity for cell-cell interactions.

Whether aggregation and blastogenesis are a manifestation of a common lectin-induced lymphocyte membrane perturbation or whether each is an independent consequence of mitogen binding is not known. Further studies to answer this question, utilising a surface membrane pharmacological approach to soluble and cellular *in vitro* antigen stimulation, in addition to mitogen activation, are in progress.

R.M.F. is a NCI fellow. This work was supported by the US Public Health Service.

RONALD M. FERGUSON*
JON R. SCHMIDTKE
RICHARD L. SIMMONS

*Department of Surgery,
University of Minnesota Hospitals,
Minneapolis, Minnesota 55455*

1 Jannossy, G., and Greaves, M. F , *Clin exp. Immun.*, **9**, 483–498 (1971).
2 Fisher, D B , and Mueller, G. C , *Biochim. biophys. Acta*, **248**, 434–448 (1971).
3 Quastel, M. R , and Kaplan, J. B , *Expl Cell Res* , **63**, 230–233 (1970)
4 Allwood, G , and Asherson, G. L , *Immunology*, **21**, 509–516 (1971).
5 Peters, J. H., and Hausen, P., *Eur. J. Biochem.*, **19**, 502–508 (1971).
6 Peters, J. H , and Hausen, P , *Eur. J. Biochem.*, **19**, 509–513 (1971).
7 Van den Berg, K J , and Betel, I., *Expl Cell Res.*, **84**, 412–418 (1974).
8 Barnett, R. E , Scott, R. E., Furcht, L. T., and Kersey, J. H., *Nature*, **249**, 465–466 (1974).
9 Andersson, J , Sjoberg, O., and Moller, G., *Transplant. Rev.*, **11**, 131–177 (1971).
10 Kondracci, E., and Milgrom, F. L., *Fedn Proc.*, **34**, 952 (1975).
11 Moorhead, J. F , Connally, J. J., and McFarland, W., *J. Immun.* (in the press).
12 Parker, C. W., Sullivan, T J , and Wedner, H. J., *Adv. Cyclic Nucleotide Res.*, **4**, 1–8 (1974).
13 Hadden, J. W., Hadden, E. M., Haddox, M. K., and Goldberg, N. D., *Proc. natn. Acad Sci. U S.A* , **69**, 3024–3027 (1972).
14 Whitney, R. B , and Sutherland, R. M , in *Proc. seventh Leukocyte Culture Conf.*, 63 (1972)
15 Godfraind, T , and Daba, A., *Br. J Pharmac* , **36**, 549–558 (1969).
16 Joanus, S. D , Miele, E , and Rubin, R. P., *Br J Pharmac Chemother.*, **31**, 319–323 (1967)
17 Seeman,·P , Chen, S S., Chau-Wong, M., and Staiman, A., *Can. J. Physiol. Pharmac.*, **52**, 526–535 (1974).
18 Wollemann, M , in *The Phenothiazines and Structurally Related Drugs* (edit. by Forrest, I S , Cau, C. J., and Usdin, E.), 731–739 (1974).
19 Ferrendelli, J A., Kinscherf, D. A., and Kipnis, D. M., *Biochem. biophys. Res. Commun.*, **46**, 2114–2117 (1972)

Temperature dependence of phenoxybenzamine effects and the adrenoceptor transformation hypothesis

ADRENOCEPTORS can be classified into two types, α or β, according to their sensitivity to agonists and antagonists. It has been claimed that the β-adrenoceptors mediating the effect of adrenaline on the contractility of frog isolated heart are changed to α-adrenoceptors by lowering the temperature from 24 to 14 °C (ref. 1). The evidence for this suggestion was that a high concentration of the α-adrenoceptor-blocking drug phenoxybenzamine inhibits the effect of adrenaline at the low temperature but not at the higher temperature, and that the frog heart binds almost twice as much phenoxybenzamine at

Table 1 Inotropic potencies of isoprenaline, adrenaline and phenylephrine (ED_{50}), and potency of propranolol (expressed as the dose ratio of isoprenaline) on the frog isolated ventricle at 24 and 14 °C

	Temperature (°C)	No. of expts	Negative log molar ED_{50} (mean±s.e.)	log Dose ratio of isoprenaline
Isoprenaline	24	7	8.48±0.31	—
Isoprenaline	14	9	8.76±0.50	—
Isoprenaline+0.34 µM propranolol	24	8	6.70±0.11	1.78
Isoprenaline+0.34 µM propranolol	14	9	6.97±0.12	1.79
Isoprenaline+3.4 µM propranolol	24	6	5.84±0.02	2.64
Isoprenaline+3.4 µM propranolol	14	6	5.85±0.02	2.91
Adrenaline	24	9	6.66±0.40	—
Adrenaline	14	9	7.37±0.62	—
Phenylephrine	24	4	5.29±0.23	—
Phenylephrine	14	5	5.46±0.22	—

Strips of frog ventricle were suspended in a solution consisting of 111 mM NaCl, 2.4 mM $NaHCO_3$, 1.9 mM KCl, 1.1 mM $CaCl_2$, 0.07 mM NaH_2PO_2, and 10 µg ml^{-1} disodium edetate. Contractions were elicited with a Grass stimulator (1–3 ms, voltage just above threshold) at a rate of 0.3 Hz (24 °C) or 0.2 Hz (14 °C) and recorded isometrically. Potencies of the agonists were determined from cumulative dose–response curves. Propranolol was present 60 min before isoprenaline was added. Potency of the antagonistic drug is expressed as the dose ratio: this is the ratio of the potency of the agonist in the presence of the antagonistic drug and in its absence. Frog experiments were carried out between the months of September and February.

the low temperature than at the higher temperature. Phenoxybenzamine, particularly in high concentration, does not, however, specifically block α-adrenoceptors, and the purpose of the present experiments was to test the hypothesis of the receptor transformation by procedures not dependent on the use of this drug.

The adrenoceptor is a descriptive term and its nature is unknown. A response is said to be mediated by α-adrenoceptors when the relative potency of a series of agonists shows that adrenaline is highest, phenylephrine is somewhat lower, and isoprenaline is very low, and when the response is susceptible to blockade by a low concentration of an α-adrenoceptor-blocking drug. A response is said to be mediated by β-adrenoceptors when the relative potency of a series of agonists shows that isoprenaline is highest and phenylephrine lowest, and when the response is susceptible to blockade by a low concentration of a β-adrenoceptor-blocking drug. The present experiments used these criteria to test the validity of the adrenoceptor transformation hypothesis; the results do not support the concept that β-adrenoceptors are changed to α-adrenoceptors at low temperature. Adenylate cyclase coupled β-adrenoceptors in frog heart[2] and erythrocytes[3] did not change with temperature.

Table 1 summarises the results of experiments to determine the inotropic potencies of isoprenaline, adrenaline and phenylephrine at 24 and 14 °C. Lowering the temperature had little effect on the potencies of the sympathomimetic drugs. Table 1

also shows the antagonism of isoprenaline by the β-adrenoceptor-blocking drug propranolol; the blocking effect of this drug was not altered by the change in temperature. It seems from these results that low temperature does not change the β-adrenoceptors mediating the inotropic effect on the frog heart.

Phenoxybenzamine is a highly lipid-soluble compound which is capable of alkylating nucleophilic centres after forming an aziridinium ion on neutralisation[4]. In isolated mammalian heart preparations nanomolar concentrations of phenoxybenzamine block α-adrenoceptors[5] and micromolar concentrations have additional effects, such as an acetylcholine antagonism at muscarinic sites[6]; an inhibition of tyramine effects[7], probably caused by blockade of neuronal uptake sites[8]; a potentiation of noradrenaline effects[7], probably the result of blockade of neuronal and extraneuronal uptake sites[9]; and a reduction in heart rate and inhibition of the chronotropic effect of noradrenaline, which is not the result of adrenoceptor blockade[10].

In agreement with previous observations[1] it was found that 7.4 µM phenoxybenzamine inhibits the response of the frog heart to adrenaline at 14 °C, that the inhibitory activity on incubation with phenoxybenzamine at low temperature persists when the temperature is raised to 24 °C, and that phenoxybenzamine had no effect when incubation was carried out in the presence of 10 µg ml^{-1} of the competitive α-adrenoceptor-

Table 2 Effect of interaction of phenoxybenzamine with acetylcholine, tyramine and noradrenaline on contractility of the frog isolated ventricle and the guinea pig isolated left atrium at 14 °C and 24 or 31 °C

	Treatment	Agonist	Temperature (°C)	log Dose ratio of agonist
Frog	0.1 µM atropine	Acetylcholine	Treatment+assay, 24	2.81
Frog	0.1 µM atropine	Acetylcholine	Treatment+assay, 14	2.58
Frog	2.9 µM phenoxybenzamine	Acetylcholine	Treatment+assay, 24	2.30
Frog	2.9 µM phenoxybenzamine	Acetylcholine	Treatment, 24; assay, 14	2.16
Frog	2.9 µM phenoxybenzamine	Acetylcholine	Treatment+assay, 14	0.79
Frog	2.9 µM phenoxybenzamine	Acetylcholine	Treatment, 14; assay, 24	0.36
Guinea pig	0.74 µM phenoxybenzamine	Acetylcholine	Treatment+assay, 31	1.96
Guinea pig	0.74 µM phenoxybenzamine	Acetylcholine	Treatment, 14; assay, 31	0.60
Guinea pig	7.4 µM phenoxybenzamine	Tyramine	Treatment+assay, 31	0.84
Guinea pig	7.4 µM phenoxybenzamine	Tyramine	Treatment 14; assay, 31	0.20
Guinea pig	7.4 µM phenoxybenzamine	Noradrenaline	Treatment+assay, 31	−1.16
Guinea pig	7.4 µM phenoxybenzamine	Noradrenaline	Treatment, 14; assay, 31	−0.08

Strips of guinea pig left atrial appendage were suspended in a solution consisting of 137 mM NaCl, 11.9 mM $NaHCO_3$. 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.36 mM NaH_2PO_4, 5.55 mM glucose and 10 µg ml^{-1} disodium edetate which was aerated with 5% CO_2 in O_2. Contractions were elicited at a rate of 1 Hz (31 °C) or 0.2 Hz (14 °C). See Table 1 for details. Incubation with phenoxybenzamine was for 40 min after which bath fluid was repeatedly changed before agonists were assayed. A positive dose ratio indicates inhibition, and a negative dose ratio potentiation, of the inotropic effect of the agonist.

blocking drug phentolamine. It was also found, however, that 10 µg ml^{-1} of the membrane stabilisers procainamide or quinidine prevented the phenoxybenzamine effect and that 7.4 µM phenoxybenzamine inhibited the effect of isoprenaline, although isoprenaline acted on β-adrenoceptors. From these results we conclude that the frog heart at low temperature is sensitive to an inhibition by phenoxybenzamine of Ca^{2+} movements which are the result of β-adrenoceptor stimulation[11]. Although the effect was persistent, it may not be based on covalent bond formation. Tissues have a high binding capacity for unchanged phenoxybenzamine[12], and lowering of temperature stabilises the intermediate aziridinium ring and greatly reduces its reactivity[4].

Other experiments measured the negative inotropic effect of acetylcholine and showed that phenoxybenzamine antagonised this effect (Table 2). Unlike atropine, phenoxybenzamine was inactive at low temperature. The behaviour of phenoxybenzamine was persistent: incubation at 24 °C led to blockade, and incubation at 14 °C did not, whether acetylcholine was assayed at 24 or 14 °C.

Studies with guinea pig atria also demonstrated the inactivity of phenoxybenzamine at low temperature. Phenoxybenzamine pretreatment at 31 °C, but not at 14 °C, inhibited the effect of tyramine and potentiated the effect of noradrenaline, and there was no inhibition of the noradrenaline effect after phenoxybenzamine treatment at 14 °C (Table 2).

Our results show that phenoxybenzamine inhibits the effect of adrenaline on the frog heart at low temperature by an action which does not involve α-adrenoceptors and that the increased tissue retention of the drug is not associated with adrenoceptor blockade. Such experiments cannot be interpreted as indicating that β-adrenoceptors are changed to α-adrenoceptors at low temperature. Our experiments to test the relative effects of agonists at high and low temperature clearly indicate that the nature of the adrenoceptors changes little with altered temperature.

B. G. BENFEY

Department of Pharmacology and Therapeutics,
McGill University, Montreal, Canada

1 Kunos, G., Yong, M. S., and Nickerson, M., *Nature new Biol*, 241, 119–120 (1973).
2 Benfey, B. G, Kunos, G., and Nickerson, M., *Br. J. Pharmac*, 51, 253–257 (1974).
3 Caron, M. G, and Lefkowitz, R J, *Nature*, 249, 258–260 (1974).
4 Rosen, G. M, and Ehrenpreis, S., *Trans. N.Y. Acad. Sci.*, ser. II, 34, 255–285 (1972)
5 Benfey, B. G., *Br J Pharmac*, 48, 132–138 (1973).
6 Benfey, B. G., and Grillo, S. A, *Br J Pharmac. Chemother.*, 20, 528–533 (1963).
7 Benfey, B. G., and Greeff, K, *Br J. Pharmac. Chemother.*, 17, 232–235 (1961).
8 Peach, M. J, and Chiu, A. T, *J Pharmac. exp Ther.*, 183, 73–79 (1972)
9 Iversen, L. L., *Br Med Bull*, 29, 130–135 (1973).
10 Adler-Graschinsky, E, Langer, S. Z., and Rubio, M. C., *J Pharmac. exp. Ther.*, 180, 286–301 (1972)
11 Triggle, D. J., *A. Rev Pharmac.*, 12, 185–196 (1972).
12 Berry, D. G, and Miller, J W, *Eur J. Pharmac.*, 31, 176–184 (1975)

Effects of sodium on solute transport between compartments in intestinal mucosal epithelium

THE uptake of amino acids and of some monosaccharides into the intestinal mucosal cell across the brush-border membranes requires sodium in the intestinal lumen[1-3]. An hypothesis of the existence in brush-border membranes of systems for transport that depend on Na—for example, the 'Na gradient' hypothesis[4]—although necessary, is not sufficient to account for all the effects of Na on epithelial transport. Whereas in *in vitro* conditions[5] the accumulation of amino acids and monosaccharides within the epithelium depends markedly on Na in the intestinal lumen, movement of these substances from the lumen into the blood in *in vivo* conditions is less dependent on Na in the lumen[6-9].

Using a vascularly-perfused preparation of frog intestine, we have shown that removal of Na from the vascular bed causes an inhibition of solute transfer into the blood that is additional to that resulting from Na-free conditions in the lumen[8,10]; here, we present further evidence to distinguish between the effects of Na on epithelial, as distinct from membrane (that is, brush-border) transport processes.

Removal of Na from the vascular perfusate decreases the extracellular space as measured by the washout into the circulation of labelled sucrose initially added to the vascular perfusate (Fig. 1). Addition of Na to the vascular perfusate increases the rate of efflux of intracellular 3–O–methyl–D–glucose (3–O–MeG) into the vascular bed, the cells initially having been loaded from the vascular side in the presence of an extracellular marker (Fig. 2a).
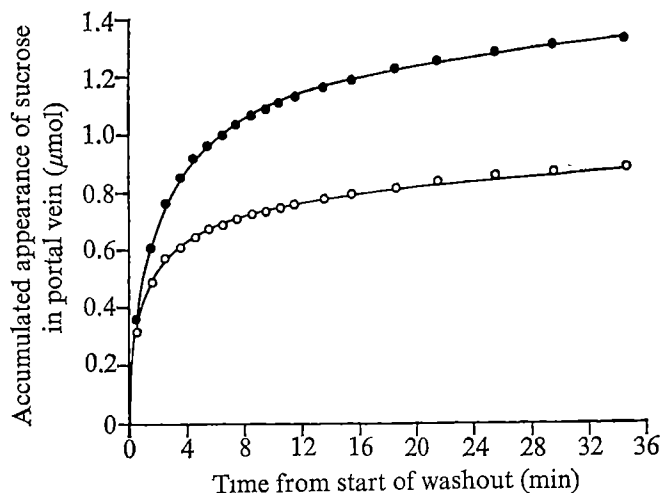

Fig. 1 Effect of presence (●) or absence (○) of sodium in the arterial infusate on the washout into the portal vein of the extracellular marker sucrose U-^{14}C labelled sucrose (1 mM; Radiochemical Centre, Amersham) was infused into the mesenteric artery for 30 min. A non-radioactive perfusion medium was abruptly switched into the arterial bed and the washout collected in 1-min fractions. Accumulated appearance of sucrose during washout is plotted against time. Choline substituted for sodium for the period of Na-free vascular perfusion; the lumen was perfused with Na-free Ringer's solution containing 5×10^{-5} M phlorizin. Control and experimental periods were carried out on the same vascularly-perfused small intestine of *R. ridibunda*[10]. Note the substantial increase in extracellular space when Na is present in the vascular perfusate: other experiments showed that neither Li nor K could substitute for Na. Vascular flow rate was 2.73 ml per min per g dry weight; that in the lumen was 0.83.

After Na-free perfusion of lumen and vascular bed, return of Na to the lumen increased the rate of efflux of 3–O–MeG from the cells into the vascular bed (Fig. 2b); although the effect of Na in the lumen was not as great as that seen with return of Na to the vascular bed, the observation that Na in the lumen may partially substitute for Na in the vascular bed strongly suggests that the effects of Na depend on events within the epithelium and not in the underlying muscle. We also found that the total quantity of 'intracellular' 3–O–MeG loaded into the cells from the circulation is increased by 43% on addition of Na to the vascular perfusate.

To investigate the effects of Na in the vascular perfusate on the translocation of intracellular amino acids into the blood, a high concentration (10 mM) of the relevant amino acids in the lumen was used to saturate the brush-border amino acid uptake systems[14]; any additional appearance of amino acid in the perfusate after subsequent addition of peptide to the lumen will then be a consequence of separate peptide uptake. We found that the removal of Na from the vascular perfusate results in a large though reversible inhibition (67%) of the transfer of amino
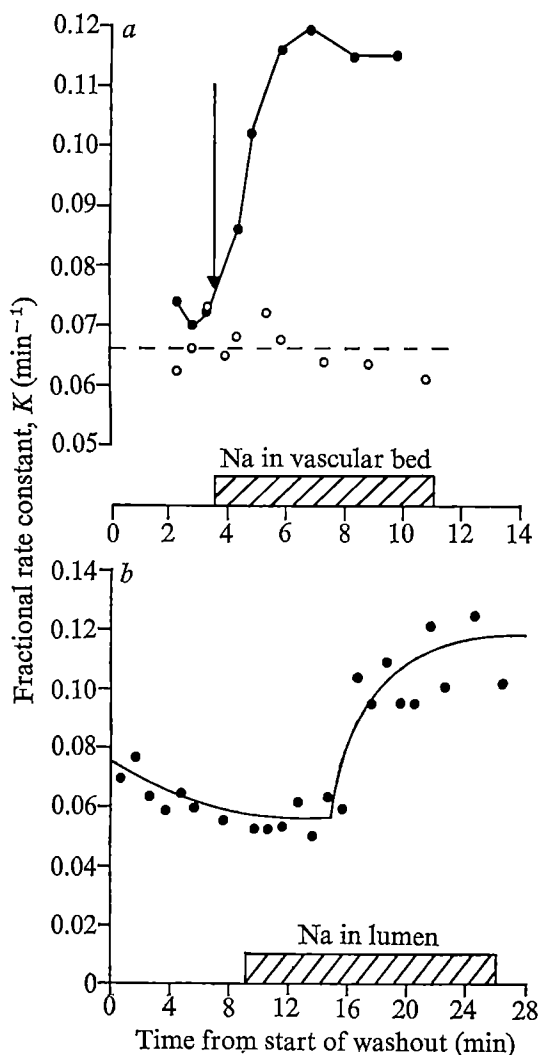
Fig. 2 *a*, Effect of introducing sodium into the vascular bed (arrow) on fractional rate constant for 'intracellular' 3-O-MeG unloading from the small intestine into the effluent collected from the portal vein. Rate of 'intracellular' 3-O-MeG appearance in the circulation was calculated from the difference in the fractional appearance during washout (see refs 11 and 12) of 1-³H-3-O-MeG and of an extracellular marker, U-¹⁴C-sucrose (both from Radiochemical Centre, Amersham) following constant infusion of both substrates at a concentration of 1 mM into the mesenteric artery for 30 min. Scintillation counting of aliquots of 1-min fractions of the effluent appearing in the portal vein was as described previously[10]. Fractional rate constants (*K*) were calculated according to Caldwell and Keynes[13]. Control Na-free (O) and experimental periods (Na where indicated, ●) were carried out on the same vascularly-perfused small intestine of *R. ridibunda*[10], thus demonstrating reversibility following Na removal. Ringer's solution was as given in ref.10 except that Li rather than K substituted for Na; however, similar findings were made when Na was replaced with choline or K. The lumen was perfused with Na-free Ringer which additionally contained 5×10^{-5} M phlorizin to ensure that any observed Na-dependent effect on 3-O-MeG movement could not have arisen at the epithelial brush-border. Vascular flow rate: 2.73 ml per min per g dry weight; luminal flow rate: 0.83. *b*, effect of introduction of Na into the lumen on fractional rate constant (*K*) for 'intracellular' 3-O-MeG unloading into the effluent appearing in the portal vein. Note that the tissue was loaded from the vascular bed and the experimental protocol was as for *a* except that K rather than Li was substituted for Na Vascular perfusate was Na-free and that in the lumen contained 5×10^{-5} M phlorizin Note delay in increase of rate constant when Na is present in lumen. This effect was consistently observed in three preparations. *R. ridibunda*, vascular flow rate: 4.56 ml per min per g dry weight; luminal flow rate: 4.90.

acids initially taken up in the form of peptide into the vascular bed. This is in marked contrast to the uptake of peptide from intestinal lumen which is independent[14] of Na in the lumen.

A further effect of Na is found on solute flux across the epithelium. Unpublished experiments (C.A.R.B.) show that the rates at which both sucrose and 2-deoxy-D-glucose (2-DOG) enter the lumen from the vascular perfusate are substantially increased (by 100% and 97%, respectively) on addition of Na to the lumen. This entry is not influenced by the presence of phlorizin in the lumen. Sucrose is confined to the extracellular space[15] and is suitable as a paracellular probe only because of the extremely low intestinal sucrase activity in the frog[8]. The route taken from the vascular bed to the lumen by 2-DOG is less certain; but whatever it may be, it clearly depends on Na, although it is insensitive to phlorizin, a known competitive inhibitor of monosaccharide transport in brush-border membranes.

The rate of appearance of 3-O-MeG and of 2-DOG in the lumen from constant vascular infusion is presented in Fig. 3. 2-DOG, lacking the hydroxyl group on C-2, is known[16] to have no affinity for the brush-border, phlorizin-sensitive, glucose uptake system, whereas 3-O-MeG behaves like glucose at the brush-border but has the advantage of not being metabolised by frog intestine[17]. It is clear that 2-DOG appears at a faster rate in the effluent from the lumen than 3-O-MeG—which initially cannot be detected—and at a time when appreciable amounts of 2-DOG are already present. A pulse of 10 mM D-glucose in the lumen results in a sudden increase in the appearance of both sugars but the effect is proportionately five times greater for 3-O-MeG. The subsequent addition of phlorizin to the lumen increases the rate of 3-O-MeG appearance in the lumen, whereas it has no effect on the movement of 2-DOG.

A simple explanation for these effects is that a significant quantity of the 3-O-MeG that enters the lumen is recaptured by
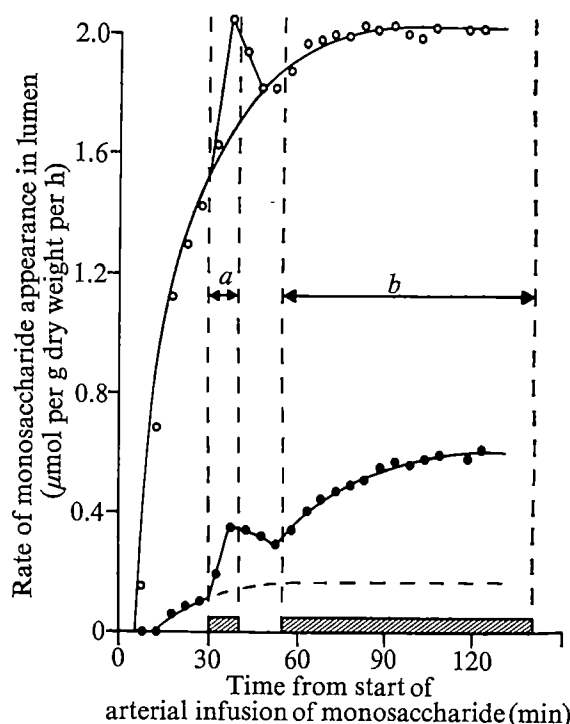
Fig. 3 Rates of appearance in the lumen of U-¹⁴C-3-O-MeG (●) and of 1-³H-2-DOG (O); (Radiochemical Centre, Amersham) after arterial infusion of both sugars at a concentration of 1 mM into the vascularly-perfused small intestine of *R. ridibunda*[10]. For the periods indicated, 10 mM D-glucose (*a*) or 5×10^{-5} M phlorizin (*b*) was added to the luminal perfusate. Vascular flow rate: 6.05 ml per min per g dry weight; luminal flow rate: 6.57.

transport systems in the brush-border. Addition to the lumen of phlorizin blocks this reuptake, thus increasing net appearance of the sugar in the lumen. We also find that glucose added to the lumen competes with 3-O-MeG for reuptake, again increasing the net appearance in the lumen of the 3-O-MeG. The absence of an effect of phlorizin and the small effect of glucose on the rate of appearance of 2-DOG in the lumen is also in accord with this explanation, although the movement of 2-DOG into the lumen involves other factors (C.A.R.B., unpublished).
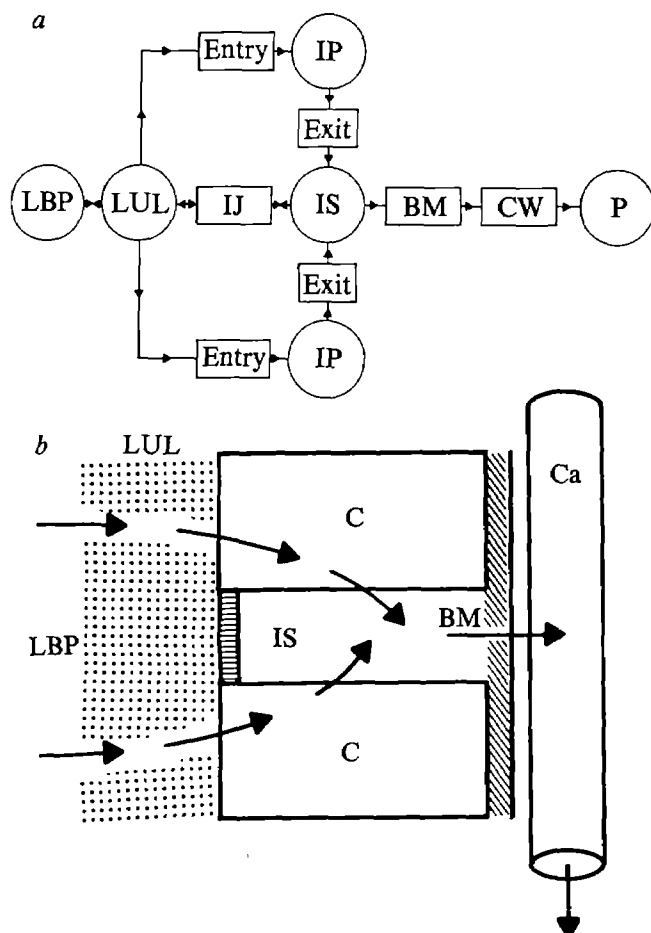
Fig. 4 *a*, Model of relationship between compartments of intestinal epithelium involved in solute transport. Rectangular box, pathway; circle, pool. Two epithelial cells and a single paracellular shunt are depicted. We suggest that Na, in addition to affecting the entry system, increases the intercellular space. LBP, Lumen bulk phase; LUL, lumen unstirred layer; IJ, intercellular junction; IS, intercellular space; IP, intracellular pool; BM, basement membrane; CW, capillary wall; P, plasma. *b*, Possible physical interpretation of the model proposed in *a*. C, Epithelial cell; Ca, capillary. Note importance of intercellular space in determining ease of access of substrate to capillary.

The presence of a compartment in the intestinal lumen adjacent to the epithelial sheet, which is unstirred[18-20] and from which specific reuptake may occur will minimise escape of substrates from the epithelium into the bulk phase of the lumen contents. Reuptake from such a compartment will be blocked by the presence in the lumen of an inhibitor of brush-border membrane transport, such as phlorizin in the case of monosaccharides, or by the presence of high concentrations of competing substrate. Axon and Creamer[21] have suggested different interpretations of similar observations.

In Fig. 4 we propose a model that may account for the effects of Na on epithelial transport. It is suggested that, in addition to the known effects on membrane transport at the brush-border (Na gradient hypothesis), Na influences the transfer of substances between compartments in the epithelium. Two of these compartments are extracellular, one intercellular within the epithelium, and the other, a stationary, unstirred layer in the lumen and adjacent to the brush-border membranes. In the light of this model, Csaky's proposal[17] that "a critical intracellular Na concentration is essential" for intestinal (sugar) transport is no longer in direct conflict with the Na gradient hypothesis. The model accounts for the fact that the presence of Na in the vascular bed is necessary for maximal rates of epithelial transport (see also ref. 22). The notion that one effect of Na on epithelial transport is to increase the size of extracellular spaces is in accord with our data on the sucrose space in the epithelium. Such an effect of Na on epithelial transport cannot be seen when the epithelium is disrupted, and has not been found[23,24].

The failure to demonstrate effects on solute transfer when the serosal Na is replaced in sheets of intestine *in vitro*[5] may depend on the presence of a substantial diffusion barrier restricting access of the bathing solution to the epithelium (intramural unstirred layer). This barrier is naturally circumvented by vascular perfusion.

We conclude that in addition to its role in membrane transport, an important function of Na in epithelial transport of solutes is in expanding extracellular spaces within the intestinal mucosa with consequential effects on the clearance of transported substrates from these spaces. The clearance may be forward into the blood stream or backwards into the unstirred layer in the lumen. Our data also suggest that the requirement for Na for this purpose can be completely fulfilled by its presence in the vascular bed, but only partially fulfilled by the addition of Na to the lumen when Na is absent from the vascular perfusate.

C. A. R. BOYD
C. I. CHEESEMAN
D. S. PARSONS

University of Oxford,
Department of Biochemistry,
South Parks Road,
Oxford OX1 3QU, UK

1 Schultz, S. G., and Curran, P. F., *Physiol. Rev.*, 50, 637–718 (1970).
2 Kimmich, G A., *Biochim. biophys. Acta*, 300, 31–78 (1973).
3 Schultz, S. G., and Curran, P. F., in *Current Topics in Membrane Transport*, 5 (edit. by Bronner, F., and Kleinzeller, A.), 225–281 (Academic, New York, 1974).
4 Crane, R. K., *Fedn Proc*, 24, 1000–1006 (1965).
5 Rinaldo, J. E., Jennings, B. L., Frizzell, R. A., and Schultz, S. G., *Am. J. Physiol.*, 228, 854–860 (1975).
6 Forster, H, in *Na Linked Transport of Organic Solutes* (edit. by Heinz, E.), 134–139 (Springer, Berlin 1972).
7 McMichael, H B., Webb, J, and Dawson, A. M., *Clin. Sci.*, 33, 135–145 (1967).
8 Parsons, D. S, and Prichard, J. S., *J. Physiol., Lond.*, 212, 299–319 (1971)
9 Bieberdorf, F. A., Morawski, S., and Fordtran, J. S., *Gastroenterology*, 68, 58–66 (1975).
10 Boyd, C. A R, Cheeseman, C. I., and Parsons, D. S., *J. Physiol., Lond.* (in the press).
11 Betz, A L., Gilboe, D. D, Yudilevich, D. L., and Drewes, L. R., *Am. J. Physiol.*, 225, 586–562 (1974).
12 Penpargkul, S., Briggs, F N., and Scheuer, J, *Proc. Soc. exp. Biol. Med.*, 147, 558–562 (1974).
13 Caldwell, P. C, and Keynes, R. D., in *Laboratory Techniques in Membrane Biophysics* (edit. by Passow, H. and Stampfli, R.), 63–68 (Springer, Berlin 1969).
14 Cheeseman, C I, and Parsons, D. S., *Biochim. biophys. Acta*, 373, 523–526 (1974).
15 Ussing, H. H, *Phil. Trans R. Soc.*, B262, 85–90 (1971).
16 Crane, R. K., *Physiol. Rev.*, 40, 789–825 (1960).
17 Csaky, T Z, *Fedn. Proc*, 22, 3–7 (1963)
18 Winne, D., *Biochim biophys. Acta*, 298, 27–31 (1973).
19 Wilson, F. A., and Dietschy, J. M., *Biochim. biophys. Acta*, 363, 112–126 (1974).
20 Dugas, M C, Ramaswamy, K., and Crane, R. K., *Biochim. biophys. Acta*, 382, 576–589 (1975).
21 Axon, A. T. R, and Creamer, B., *Gut*, 16, 99–104 (1975).
22 Fisher, R. B., and Gardner, M. L. G., *J. Physiol., Lond*, 241, 235–260 (1974).
23 Bihler, I., and Cybulsky, R., *Biochim. biophys. Acta*, 298, 429–437 (1973).
24 Murer, H., Hopfer, U., Kinne-Saffran, E., and Kinne, R., *Biochim. biophys. Acta*, 345, 170–179 (1974).

Synthesis and conformations of hypothalamic hormone releasing factors: two QRF-analogues containing backbone N-methyl groups

SINCE its isolation and characterisation[1,2], the hypothalamic hormone-releasing factor TRF (pGlu–His–Pro–NH$_2$) has been the subject of several conformational studies. Most investigations have indicated that the steric characteristics of the three adjacent rings strongly limit the backbone conformational flexibility and support a preference for an extended structure. The importance of each functional group in the molecule in terms of biological potency has been systematically investigated (for review, see ref. 3). With one exception (methylation of the histidyl side chain in the N$^\tau$ position) all the chemical modifications which have been made to date were accompanied by a substantial loss of biological activity. We have shown[4] that most of these modifications do not significantly affect the preferred backbone conformation of the hormone, emphasising the critical role of each functional group in TRF for optimal binding to the receptor sites.

To investigate the 'active' conformation of the hormone we tried to influence the conformational equilibrium of the tripeptide (without affecting its functional characteristics) by preparing an analogue which contains a methylated peptide group between the pyroglutamyl and histidyl residues, [N$^\alpha$MeHis^2]TRF, and a second analogue in which the prolyl residue was substituted by N$^\alpha$methylalanyl, [N$^\alpha$MeAla^3]TRF. This latter compound was previously suggested by Burgess *et al.*[5]. From model manipulations it was expected that the first modification would cause a further stabilisation of the molecule in an extended form. Steric interactions between the methyl group and the carbonyl group of the histidyl–prolyl peptide residue, on the one hand, and the β-methylene protons of the histidyl side chain on the other, are expected to affect

slightly the values for the backbone rotational angles of the extended form (φ_2, ψ_2) as well as the rotational isomerism of the histidyl side chain (χ_1, χ_2). This modification also prevents the formation of an intramolecular hydrogen bond between the pyroglutamyl–histidyl peptide proton and the N$^\pi$-nitrogen of the imidazole ring, and therefore provides an adequate test of the hairpin turn model[6] proposed[7] as the active three-dimensional structure of the hormone. In the second modification, replacement of the prolyl residue by N$^\alpha$methylalanyl introduces an additional degree of freedom for the rotational angle φ_3. For a typical φ_3 value, however, the N$^\alpha$methylalanyl residue sterically simulates the prolyl residue reasonably well.

Both analogues were synthesised by the solid-phase technique using a benzhydrylamine resin of 0.15 mEq g^{-1} substitution as support. The amino acid residues were introduced as Boc-derivatives (with the exception of ZpGluOH) and coupled in threefold excess in methylenechloride, using dicyclohexylcarbodiimide as coupling agent. The tripeptides were cleaved from the resin by the action of double-distilled hydrofluoric acid at 0 °C over a 45-min period.

The amino acid derivative N$^\alpha$methylhistidine was prepared as described previously[8]. Histidine hydrochloride was treated with a threefold excess of benzaldehyde in the presence of N,N-dimethylbenzylamine as base, to give N$^\alpha$benzylhistidine which was then methylated by refluxing a formic acid solution of the compound in the presence of an equimolar amount of formaldehyde for 15 min. After cooling, the solution was hydrogenated directly using 10% palladium–charcoal as catalyst. The crude product isolated as the monohydrochloride, was recrystallised twice from water–dimethylsulphoxide and once from water–ethanol (melting point 265–267 °C) and its purity confirmed by thin layer chromatography. It was characterised by mass spectroscopy (peak matching). The N$^\alpha$Me isomer can easily be differentiated from the N$^\tau$ isomer and from the bridged spinacin side product by ion-exchange chromatography as monitored on an amino acid analyser and by mass spectroscopy. The N$^\alpha$methylhistidine was then treated with an excess of *tert*-butyloxycarbonyl fluoride in a dioxane–water mixture at 0 °C and pH 8.5 until all the starting
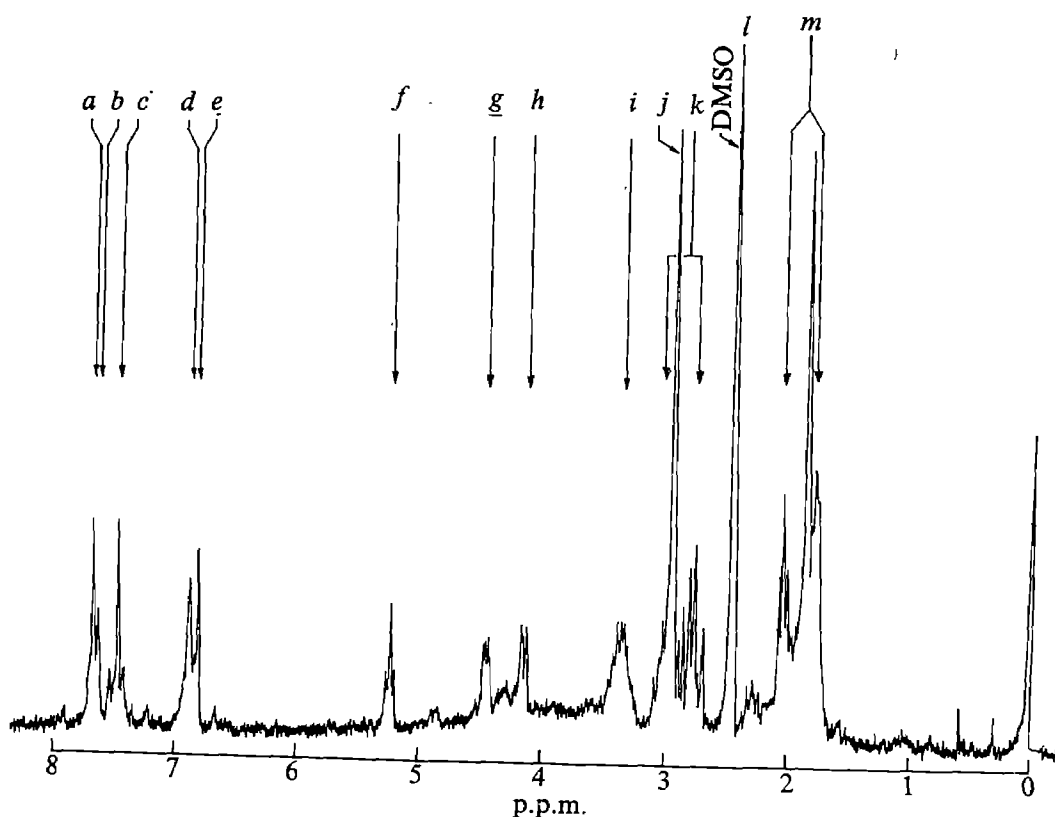

Fig. 1 NMR spectrum (220 MHz) of [N$^\alpha$Me-His^2]TRF acetate in DMSO$_{d_6}$ at 22 °C. *a*, pGlu-NH; *b*, ProCONH$_2$ anti-*trans*; *c*, Im-C^{2}H; *d*, ProCONH$_2$ *syn-trans*; *e*, Im-C^{4}H; *f*, His-C$^\alpha$H; *g*, Pro-C$^\alpha$H; *h*, pGlu-C$^\alpha$H; *i*, Pro-C$^\delta$H$_2$; *j*, His-N$^\alpha$Me; *k*, His-C$^\beta$H$_2$; *l*, DMSO; *m*, pGlu-C$^\beta$H$_2$, C$^\gamma$H$_2$; Pro-C$^\beta$H$_2$, C$^\gamma$H$_2$.

materialwastransformedintoN$^\alpha$Me, N$^\alpha$Boc, N^{1m}BocHisO$^-$Na$^+$. After concentration of the solution *in vacuo*, it was acidified to pH 3.5 and extracted with a mixture of 20% ethyl acetate in ether, from which the *bis*Boc derivative was obtained as a colourless oil.

After cleavage from the resin, the tripeptides were purified by chromatography on carboxymethyl cellulose eluting with a 0.04 M solution of ammonium acetate, and then by partition chromatography on Sephadex G25 in a butanol–acetic acid–water (4:1:5) system. The products were found to be pure by thin layer chromatography and were characterised by amino acid analysis, mass spectroscopy (peak matching) and nuclear magnetic resonance (NMR) spectroscopy.

The biological potencies of the two analogues were determined as described previously[9,10]. The *in vivo* bioassay is based on the ability of TRF or active homologues to release thyroid-stimulating hormone (TSH) from the anterior pituitary, thereby increasing the blood level of ^{125}I-labelled thyroid hormones in appropriately prepared mice. The potencies relative to pGlu–His–Pro–NH$_2$ are as follows: [N$^\alpha$MeAla^3]TRF =4.5±1.3%; [N$^\alpha$MeHis^2]TRF=115±8%. [N$^\alpha$MeHis^2]TRF was assayed *in vivo* three times giving an average potency of 115% which is not statistically different from that of TRF; this analogue was also found to be indistinguishable from TRF in its ability to stimulate *in vitro* TSH secretion by cultured rat pituitary cells.

The 220-MHz NMR spectrum of [N$^\alpha$MeHis^2]TRF in DMSO$_{d_6}$ is shown in Fig. 1. The following differences from the spectrum of TRF were observed: an upfield shift of the anti-*trans* carboxamide resonance of 0.4 p.p.m.; a quasi-disappearance of the non-equivalence of the Pro-C^5 H$_2$ resonances; and an upfield shift of about 0.1 p.p.m. of the Im-C^{4}H resonance. These differences strongly suggest that the imidazole–carboxamide interaction which was found in TRF (in DMSO)[4] is destabilised in the case of [N$^\alpha$MeHis^2]TRF. This is further supported by the fact that the vicinal coupling constants in the C$^\alpha$-C$^\beta$ fragment of the histidyl side chain, which were non-equivalent in TRF (J_{AX}=5.3 Hz; J_{BX}=7.6 Hz), are equivalent in [N$^\alpha$MeHis^2]TRF (J_{AX}=J_{BX}=7.0 Hz). In terms of the relative populations of the side-chain rotamers, this means that the two *gauche* conformers which were unequally populated in TRF (b=0.42, c=0.22), are equally populated in [N$^\alpha$MeHis^2]TRF (b=0.37, c=0.37).

A quantitative interpretation of the ABX system of the histidyl side chain in [N$^\alpha$MeAla^3]TRF was not possible because of overlapping with the methyl resonance. The resonance positions of the anti-*trans* carboxamide proton and of the imidazole C4-proton in this analogue are the same as in TRF, however, which suggests that the imidazole–carboxamide interaction is also favoured in [N$^\alpha$MeAla^3]TRF dissolved in DMSO.

The differences between the NMR spectra of [N$^\alpha$MeHis^2]TRF and TRF itself can be explained by a change in the preferred conformation of the histidyl side chain and a small distortion of the preferred backbone conformation. The high biological potency of this analogue in *in vivo* as well as in *in vitro* tests clearly shows that the steric constraints which were introduced on methylation of the pyroglutamyl–histidyl peptide bond did not affect the binding of the hormone to its receptor sites. For the same reason, the hypothetical hydrogen bond between the pyroglutamyl–histidyl peptide proton and the N$^\pi$ of the imidazole ring does not seem to be a conformational requirement for hormone–receptor interaction.

B. DONZEL
M. GOODMAN

Department of Chemistry,
University of California, San Diego,
La Jolla, California 92037

J. RIVIER
N. LING
W. VALE

The Salk Institute for Biological Studies,
La Jolla, California 92037

1 Burgus, R., Dunn, T F., Desiderio, D., Ward, D., Vale, W., and Guillemin, R., *Nature*, 226, 321–325 (1970).
2 Nair, R M. G., Barrett, J F , Bowers, C. Y., and Schally, A. V , *Biochemistry*, 9, 1103–1106 (1970).
3 Vale, W., Grant, G., and Guillemin, R., in *Frontiers in Neuroendocrinology* (edit. by Ganong, W F., and Martini, L), 11 (Oxford University Press, New York and London, 1973).
4 Donzel, B , Rivier, J., and Goodman, M., *Biopolymers*, 13, 2631–2647 (1974).
5 Burgess, A W., Momany, F. A , and Scheraga, H. A , *Proc. natn. Acad. Sci. U.S A.*, 70 (5), 1456–1460 (1973).
6 Blagdon, D., Rivier, J., and Goodman, M., *Proc. natn. Acad. Sci. U.S.A.*, 70 (4), 1166–1168 (1973).
7 Grant, G., Ling, N , Rivier, J., and Vale, W., *Biochemistry*, 11 (16), 3070–3073 (1972).
8 Reinhold, V. N., Ishikawa, Y., and Melville, D. B., *J. med. Chem.*, 11, 258–260 (1968).
9 Vale, W., Dunn, T , Burgus, R., and Guillemin, R., *Hormones*, 2, 193–203 (1971)
10 Vale, W , Grant, G., Amoss, M., Blackwell, R., and Guillemin, R., *Endocrinology*, 91, 562–572 (1972).

Transformation of human cells in culture by N-methyl-N'-nitro-N-nitrosoguanidine

THE development of *in vitro* cell culture systems in which healthy cells can be transformed (converted to malignant cells) by chemicals, and detailed studies of the interaction of chemical carcinogens (or their activated forms) and cells, have aided the study of chemical carcinogenesis. *In vitro* chemical transformation of various rodent cells has been well established[1–3]. Many attempts have been made to transform various cultured normal or genetically abnormal human cells with chemical carcinogens without success[4,5]. We have tried to transform human cells from both normal and abnormal individuals and as has been the experience of others, have failed to observe any changes in such cultures. Therefore, the possibility of using continuous lines of human sarcoma cells for chemical transformation was investigated, since certain human sarcoma cell lines are susceptible to transformation by both DNA and RNA tumour viruses[6–8]. This communication reports results of experiments showing that human osteosarcoma clonal cells can be transformed *in vitro* by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a known potent carcinogen[9,10] and that these transformed cells produced tumours when injected into NIH nude athymic mice.

A human osteosarcoma clonal cell line designated as TE-85 clone F-5 originally established by McAllister *et al.*[11], was obtained from Naval Biomedical Research Laboratory, Oakland, California. The line had cytological and karyological characteristics similar to those of the parent tumour: it grew to high saturation density, formed colonies in agar medium and was aneuploid but did not produce tumours in anti-thymocyte serum-treated hamsters or mice. This line was also found to be highly susceptible to transformation by a feline sarcoma virus[7]

Table 1 Morphological alterations of human cells* treated with MNNG

Passage	MNNG	Morphological alterations			Growth medium
		MNNG (0.1 µg ml^{-1})	MNNG (0.01 µg ml^{-1})	DMSO (0.5%)	
0	MNNG treated for 7 d				
1	14	−	−	−	−
4	35	−	−	−	−
6	49	±	−	−	−
7	55	+	+	−	−
9	72	+	+	−	−

*The human osteosarcoma clonal line (TE-85, clone F-5) at the 31st subculture.

Table 2 The karyology of human osteogenic sarcoma cells (TE-85 clone F-5) and chemically-treated TE-85 clone F-5 cells

Treatment	Morphology	Range of no. of chromosomes	Poly-ploidy (%)	Giemsa conventional 'markers'
		Modal no.		
Control (P-13)*	Epithelial-like (polygonal) no foci	46–53 / 51	38	2–3 Exceptionally long subtelocentrics and 1–2 minutes
MNNG 0.01 µg ml⁻¹ (P-13)	Distinct foci of 'piled-up' (+++), refractile cells	72–80 / 73	99	2–3 Exceptionally long subtelocentrics and 1–2 minutes
MNNG 0.1 µg ml⁻¹ (P-13)	Distinct foci of 'piled-up' (+), refractile cells	43–49 / 49	5	2–3 Exceptionally long subtelocentrics and 1–2 minutes

*Number of subcultures after chemical or DMSO treatment.

and the Kirsten sarcoma virus (KiSV)[8,12]. Growth and maintenance medium consisted of Eagle's minimal essential medium (MEM) with 10% foetal bovine serum (FBS), 2 mM glutamine, 100 units of penicillin and 100 µg of streptomycin ml⁻¹ (MEM+10% FBS).

One day after plating 2×10^5 cells ml⁻¹ from the 31st subculture in Falcon plastic Petri dishes, the medium (MEM+10% FBS) was removed and replaced with media containing MNNG at various concentrations (5.0 µg ml⁻¹ to 0.01 µg ml⁻¹) in 0.5% dimethyl sulphoxide (DMSO). The control medium contained 0.5% DMSO. After 7 d treatment with the carcinogen, the cultures were washed, fed again with carcinogen-free growth medium and subsequently passaged by trypsin treatment every 7 d. When changes in morphology and growth patterns appeared, some cultures were fixed in alcohol and stained with Giemsa for further microscopy. Both transformed and untransformed cultures were established as continuous cell lines.

In the MNNG-treated cultures, morphological alterations of cells and abnormal pattern of growth were noted in the sixth subculture, 55–59 d after treatment (Table 1); a similar change was not observed in the control cells treated with DMSO or in the untreated human cells. Doses of 1.0 µg MNNG ml⁻¹ or greater were lethal. The change was first observed in the cells treated with 0.1 µg MNNG ml⁻¹; however, the alteration observed in the MNNG (0.01 µg ml⁻¹) treated cells became more pronounced after further subcultivation.

The morphological changes observed in these cultures were similar to those observed in a previous study in which KiSV alone transformed TE-85 clone F-5 cells[8,12]. Foci of transformed cells consisted largely of criss-crossed, randomly oriented, spindle-shaped cells with nuclear and cytoplasmic overlapping which stained heavily with Giemsa (Fig. 1a and b). In contrast, the cellular morphology remained unchanged in the untreated, control human cell line, which continued to grow in monolayers of normal-appearing, fibroepithelial-like cells (Fig. 1c) for 18 subcultures during the 112 d after treatment. An increased growth rate (double that of controls) was observed in the MNNG-treated cells, and they had to be subcultured much more frequently than untreated cells.

The relationship between the transformed and control cells was established by chromosome analysis (Table 2). Untreated cells had remarkable marker chromosomes by conventional staining; the same markers were noted in cells of the transformed cultures. Trypsin–Giemsa banding of cells revealed banded markers previously reported in the parental untreated cells, TE-85 clone F-5 (ref. 13). The fluctuation in polyploidy does not seem to be related to the transformation state, since cells near diploid as well as near triploid were involved.

The MNNG-treated cells, as well as untreated control cells, were tested for their ability to transplant and produce tumours in nude athymic mice. Cells were trypsinised, centrifuged, suspended in growth medium, and inoculated subcutaneously (Table 3). All the inoculated nude mice given 5×10^6 cells from MNNG (0.01 µg ml⁻¹) treated cells developed tumours within 2 weeks at the site of inoculation. The tumours were poorly-differentiated sarcomas. A few of the cells were producing a matrix which had some resemblance to osteoid. Moreover, three of five mice given cells from MNNG (0.1 µg ml⁻¹) treated cells produced small, persistent nodules. The nodule tumours resembled osteosarcoma and there was considerable matrix resembling osteoid around many of the cells. No tumours developed over a period of 50 d in a group of nude mice inoculated subcutaneously with the same number of cells from the untreated cultures (Table 3). The tumours were progressive and transplantable. Cells established from the tumours resembled the MNNG-treated cells (Fig. 1d).

The human osteosarcoma clonal cells treated with MNNG had the following properties: (1) The MNNG-treated cells were morphologically altered and grew as randomly oriented multilayers; (2) the treated cells showed increased growth rates; (3) when inoculated into nude athymic mice, one concentration (0.01 µg ml⁻¹) produced subcutaneous tumours and another (0.1 µg ml⁻¹) produced small persistent tumour nodules. It thus seems clear that we have demonstrated the transformation *in vitro* by MNNG of human cells from a continuous cell line. Other continuous cell lines of hamster origin, BHK-21 (ref. 14), of mouse origin, 3T3 (ref. 15) and BALB/c 3T3 (ref. 16), are all susceptible to transformation by

Table 3 Tumours produced in NIH nude mice by human osteogenic sarcoma cells (TE-85, clone F-5) obtained by treatment with MNNG*

Treatment	No. of subcultures after MNNG or DMSO treatment	No. of tumours / No. inoculated	Remarks	Pathology
MNNG (0.01 µg ml⁻¹)	18	†5/5 (12)‡	Progressive, transplantable tumours	Poorly differentiated sarcomas
MNNG (0.1 µg ml⁻¹)	18	†3/5 (12)	Small, persisting nodules	Osteosarcomas
Control	18	0/5		

*5×10^6 cells inoculated per mouse.
†Tumours were sectioned and diagnosed by Dr D. N. Taylor. Tumours also re-established in tissue cultures.
‡Day tumour first noted.
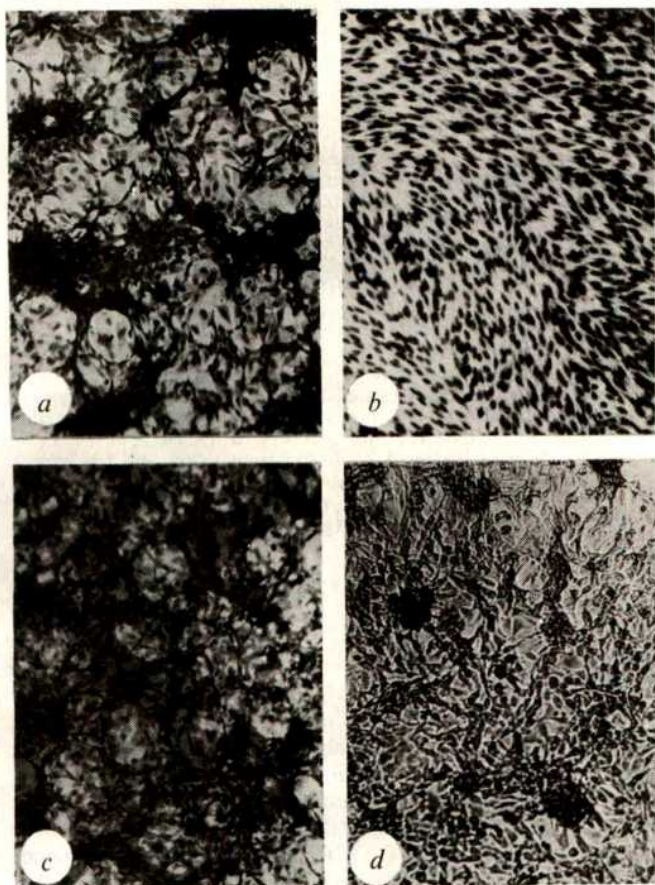
Fig. 1 Human osteosarcoma clonal (TE-85, Clone F-5) cells treated with MNNG for 7 d followed by seven subcultures in nutrient medium. Giemsa stain ×39. Note the morphological alterations in human cells treated with 0.01 µg ml⁻¹ and 0.1 µg ml⁻¹ of MNNG: criss-crossed, spindle-shaped cells with nuclear and cytoplasmic overlapping (*a* and *b*). *a*, Human cells treated with 0.01 µg ml⁻¹ MNNG; *b*, human cells treated with 0.1 µg ml⁻¹ MNNG; *c*, human cells (0.5% DMSO); *d*, typical field of culture originating from a primary tumour induced by the MNNG (0.01 µg ml⁻¹) treated human cells.

both DNA and RNA viruses[6] as well as by chemical carcinogens[9]. We confirmed the *in vitro* human sarcoma cell transformation by MNNG and also found that a carcinogenic polycyclic aromatic hydrocarbon, 7,12-dimethylbenz(a)anthracene induced transformation in the human osteosarcoma (TE-85, clone F-5) cell system described here (our unpublished work). Although unfortunately an aneuploid sarcomatous cell line had to be used for this investigation, in view of the fact that little, if any, successful *in vitro* transformation of normal human cells by chemical carcinogens[4,5] had been reported, the system presented in this report may offer an initial advancement in this direction.

It is interesting to note that the chemically altered human cells like the KiSV-transformed TE-85 cells[12] produced tumours when implanted subcutaneously into NIH nude athymic mice whereas control, untransformed cells did not. Although human TE-85 clone F-5 cells which we used in our experiment had cytological characteristics similar to those of the parent tumour[11], they did not form subcutaneous tumours in anti-thymocyte serum (ATS) treated hamsters or ATS-treated mice and did not induce fibrinolytic activity. Subcutaneous inoculation of the original lines TE-85 clone F-5, both from the NBRL repository and our control culture never produced nodules or tumours following implantation of 5 × 10⁶ viable cells. In addition, it is interesting that KiSV-transformed human TE-85 clone F-5 cells induce fibrinolytic activity and yield tumours in ATS-treated hamsters (R. M. McAllister,

personal communication). Studies are in progress to determine whether the chemically altered cells do induce tumours in ATS-treated hamsters and also whether they induce fibrinolytic activity like KiSV-transformed cells.

MNNG, a known potent carcinogen and mutagen that does not require metabolic activation, has been repeatedly demonstrated to transform mammalian cells[9,10]. Henderson *et al.*[17] have reported an increased transformation frequency of human peripheral lymphocytes following treatment with MNNG, possibly by activation of Epstein–Barr virus. Attempts to activate type C virus or virus expression in MNNG-altered cells are in progress.

J. S. RHIM
D. K. PARK

Microbiological Associates,
Bethesda, Maryland 20014

P. ARNSTEIN
R. J. HUEBNER
E. K. WEISBURGER

National Cancer Institute,
Bethesda, Maryland 20014

W. A. NELSON-REES

University of California,
*Naval Biomedical Research Laboratory,*
Oakland, California 94625

1 Berwald, Y., and Sachs, L., *J. natn. Cancer Inst.*, 35, 641–661 (1965).
2 DiPaolo, J. A., and Donovan, P. J., *Expl Cell Res.*, 48, 361–377 (1967).
3 Chen, T. T., and Heidelberger, C., *J. natn. Cancer Inst.*, 42, 915–925 (1969).
4 Leith, R. S., and Hayflick, L., *Proc. Am. Ass. Cancer Res.*, 15, 86 (1974).
5 Dietz, M. H., and Flaxman, B. A., *Cancer Res.*, 31, 1206–1209 (1971).
6 MacPherson, I., *Adv. Cancer Res.*, 13, 169–215 (1970).
7 McAllister, R. M., *et al.*, *Nature new Biol.*, 230, 279–282 (1971).
8 Klement, V., Freedman, M. H., McAllister, R. M., Nelson-Rees, W. A., and Huebner, R. J., *J. natn. Cancer Inst.*, 47, 65–73 (1971).
9 DiPaolo, J. A., Takano, K., and Popescu, N. C., *Cancer Res.*, 32, 2686–2695 (1972).
10 Heidelberger, C., *Fedn Proc.*, 32, 2154–2161 (1973).
11 McAllister, R. M., *et al.*, *Cancer*, 27, 397–402 (1971).
12 Rhim, J. S., Cho, H. Y., and Huebner, R. J., *Int. J. Cancer*, 15, 23–29 (1975).
13 Nelson-Rees, W. A., Flandemeyer, R. R., and Hawthrone, P. K., *Int. J. Cancer*, (in the press).
14 MacPherson, I., and Stoker, M. G., *Virology*, 16, 147–151 (1962).
15 Todaro, G. J., and Green, H., *J. Cell Biol.*, 17, 299–313 (1963).
16 Aaronson, S. A., and Todaro, G. J., *Science*, 162, 1024–1026 (1968).
17 Henderson, E. E., Norin, A. J., and Strauss, B. S., *Cancer Res.*, 35, 358–363 (1975).

Detection of DNA complementary to pathogenic viroid RNA in exocortis disease

A LOW molecular weight (about 10⁵ daltons) free RNA has been identified as the pathogenic agent of the exocortis disease of citrus (CEV) by Semancik and Weathers[1]. This minimal 'infectious' RNA (viroid), characterised by a highly ordered structure rich in G–C base pairs[2] does not contain polyadenylate sequences[3] nor can it be translated *in vitro*[4]. The CEV–RNA induces the identical symptom expression in tomato and potato as the spindle tuber viroid[5], thus supporting the class relationship of these unique pathogenic RNAs. The limited genetic potential of these extremely low molecular weight pathogenic RNAs lends credence to the suggestion of host-dependent synthesis[6] and the homology of the pathogenic CEV–RNA with the host genome. This relationship is supported by the detection of a CEV-like DNA, reported here, presumably resulting from the action of host polymerase[7]. The final pathogenic response induced by the viroid RNA might then reflect simply an aberrant expression of the host genome. These functions, resulting in pathogenic RNA synthesis by means of a DNA intermediate as well as the intimate interaction with
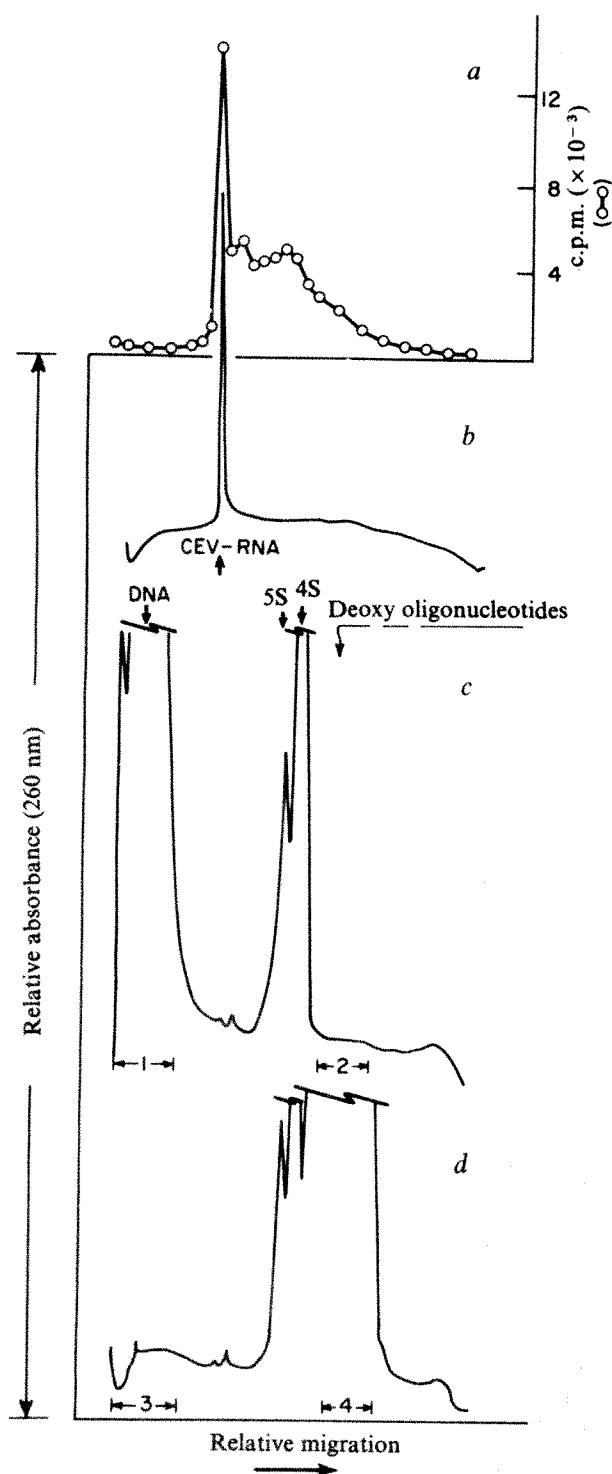
Fig. 1 Electrophoresis on 5% polyacrylamide gels of ^{125}I-labelled CEV–RNA (*a*) as iodinated from a purified preparation as shown in the A_{260nm} scanning pattern (*b*) and a DNA-rich preparation from exocortis-infected *Gynura aurantiaca* before (*c*) and after (*d*) treatment with pancreatic DNase I. CEV–RNA was purified as previously reported[2] and iodinated with ^{125}I as reported[9] to a specific activity of about 10[7] d.p.m. µg^{-1} RNA. DNA-rich preparations were derived from extraction of fresh tissue with cold phenol and winding high molecular weight DNA from a 2 M LiCl-soluble fraction after addition of three volumes of ethanol. After sedimentation in 1.73 g cm^{-3} CsCl for 64 h at 10 °C and 35,000 r.p.m. in a Beckman 65 rotor, the DNA in the supernatant was again removed by winding on to a glass rod after removal of CsCl by dialysis and addition of three volumes of ethanol. Final samples were resuspended and dialysed against TKM buffer (0.01 M Tris, 0.1 M KCl, 10^{-4} M MgCl, *p*H 7.4). Sample (*d*) was treated with 15 µg ml^{-1} pancreatic DNase I at room temperature for 15 min immediately before electrophoresis[10]. Gels were scanned in a Beckman ACTA CII spectrophotometer, sliced on a Mickle model 140 gel slicer, and either incubated for 48 h at 37 °C in omnifluor toluene scintillator +3% protosol before counting, or eluted overnight in distilled H_2O for recovery of nucleic acid species. Samples from gel areas 1,2,3,4, indicated in (*c*) and (*d*) were hybridised in 40% formamide with ^{125}I-CEV–RNA according to Friedrick and Feix[8] with the RNase-resistant c.p.m. as follows: Area 1, 958; 2, 17; 3, 62; 4, 375.

prepared from healthy and infected gynura and tomato (*Lycopersicon esculentum* Mill, cv. Rutgers) as well as CEV-resistant tobacco (*Nicotiana tabacum*) and cowpea (*Vigna unguiculata* (L.)) Walp. cv. Early Ramshorn. Table 2 indicates that ^{125}I-CEV–RNA specifically hybridises only with DNA extracted from diseased tissues. The existence of a CEV-like DNA is further supported by the reduction in activity in the presence of added carrier CEV–RNA as well as the dependence of RNase resistant activity on DNA concentration. Pre-treatment with DNase, however, could not completely reduce the count level to background, presumably because of the ability of DNA fragments to hybridise with the ^{125}I-CEV–RNA, as suggested by gel electrophoresis (Fig. 1*c*, *d*).

Verification of the presence of a CEV-like DNA was made by analysis of DNA preparations in 5% polyacrylamide electrophoresis and CsCl equilibrium sedimentation. The quality of the isolated CEV–RNA preparation before and after iodination is shown in the gel patterns of Fig. 1*a* and *b*. *In vitro* labelling with ^{125}I as reported by Prensky *et al.*[9] results in the degradation of a portion of the CEV–RNA population to approximately 2/3 and 1/3 fragments (Fig. 1*a*). Since DNA preparations were not RNase treated, contaminating RNA species, principally 4S and 5S RNA, are observed even following successive windings from 75% ethanol medium coupled with centrifugation in 1.73 g cm^{-3} CsCl for 65 h.

The position of DNA on the 5% gels (Fig. 1*c*) was identified by the action of DNase (Fig. 1*d*). Areas of these gels (indicated in Fig. 1*c* and *d* as areas 1, 2, 3, 4) containing the region of native DNA (area 1) and the oligodeoxynucleotides (area 4) were eluted and concentrated. Hybridisation with ^{125}I-CEV–RNA indicated the presence of the CEV-complement in the region of native DNA as well as the DNase-produced product. This further supports the existence of a CEV-like DNA species but does not obviate the possible existence of an RNA moiety trapped in the DNA matrix which is complementary to CEV–RNA and demonstrates a distinctly different mobility in a DNase-treated preparation.

For this reason, DNA preparations were subjected to CsCl equilibrium sedimentation (Fig. 2), and samples recovered from the DNA band as well as any pelleted RNA, were hybridised with ^{125}I-CEV–RNA. A CEV-like DNA is contained in the CsCl banded DNA. No evidence was detected for RNA–RNA hybridisation of CEV–RNA to either pelleted RNA or tRNA-like species concentrated at high CsCl densities. Sedimentation in 56% (w/w) CsCl (Fig. 2) indicated heterogeneity in the DNA preparations from healthy gynura

the host cell, offer striking parallels to the oncogenic viral processes operating at a minimal level.

In an attempt to localise and identify the CEV–RNA template, total nucleic acids extracted from subcellular fractions of infected *Gynura aurantiaca* were hybridised[8] with iodinated ^{125}I-CEV–RNA[9] (Table 1). The most significant level of RNase-resistant ^{125}I-CEV–RNA binding was observed with nucleic acid from the 250-g pellet which contained the bulk of the nuclear DNA as judged by the diphenylamine test. This fraction also contained pathogenic CEV–RNA as indicated by the relative 'infectivity' assay.

To determine whether a CEV-like DNA was specific to exocortis disease tissues, high molecular weight DNAs were
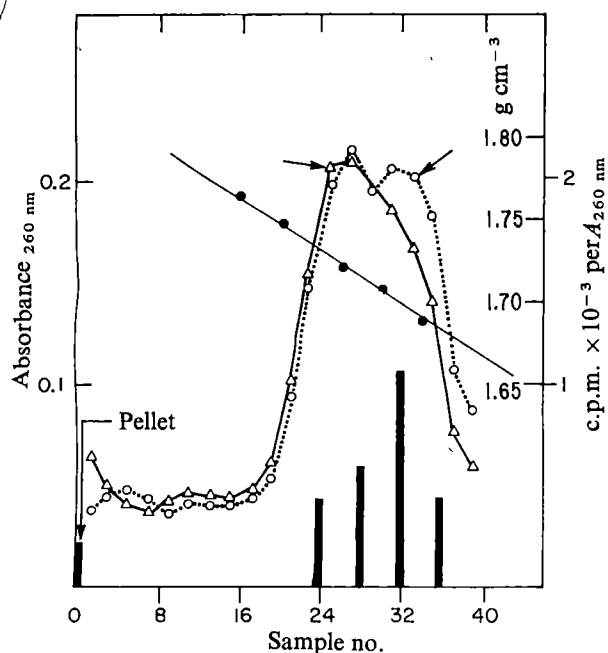
Fig. 2 Equilibrium sedimentation of DNA-rich preparations from healthy and exocortis-infected gynura in 56% (w/w) CsCl and 10 °C in the SW 50 Beckman rotor for 65 h in 0.01 M Tris, pH 8.0. The tubes were fractionated by bottom puncturing and 30–40 samples collected from 5-ml columns. Alternate samples were diluted to 1 ml with TKM buffer and A_{260nm} recorded. Densities were determined on undiluted samples by refractive index. Samples to be used for hybridisation with ^{125}I-CEV–RNA were dialysed overnight against 0.1 × TKM buffer followed by lyophilisation and resuspension in 100 μl H_2O and hybridised as in Fig. 1. The specific activity of c.p.m. per A_{260nm} (■■■■) records the recovery of RNase-resistant ^{125}I-CEV–RNA in selected samples included sedimented RNA. △, Healthy plants; ○, infected.

with the presence of a fraction of lower density than the main-band DNA. This light satellite DNA is more easily resolved in DNA preparations from diseased gynura and demonstrates a greater affinity for CEV–RNA than mainband DNA. Since the CEV–RNA is 58% G+C (ref. 2) the putative DNA complement would be correspondingly high in A+T and therefore characterised by relatively low intrinsic buoyant density. Furthermore, the enhanced resolution of the light DNA in preparations from diseased tissue suggests the pro-portionately higher concentration of the light DNA possibly induced by the pathogenic activity of CEV–RNA.

These data indicate the existence of a DNA sequence in diseased tissue with an affinity for the CEV–RNA resulting in an RNase-resistant RNA–DNA hybrid. Previous studies[1] demonstrated that the pathogenic RNA contained in 2 M LiCl supernatant preparations from exocortis disease tissue could be localised after sedimentation in Cs_2SO_4 in a region inter-mediate to dsRNA (1.60 g cm⁻³) and tRNA (1.51–1.58 g cm⁻³). If the CEV–RNA became associated with relatively high molecular weight species of DNA then a shift of the pathogenic activity to lower density Cs_2SO_4 might be expected.

Figure 3b shows the separation of single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), tRNA and DNA along with the distribution of CEV–RNA by the relative infectivity index. Aliquots of the sample (Fig. 3b) were sub-jected to conditions of melting and rapid (Fig. 3c) or slow (Fig. 3a) cooling. A shift in the density of the DNA sample (Fig. 3a, c) from 1.40 g cm⁻³ to 1.44 g cm⁻³ resulted from the melting at 100 °C for 10 min. The demonstration of patho-genic activity was not affected by the thermal treatment,

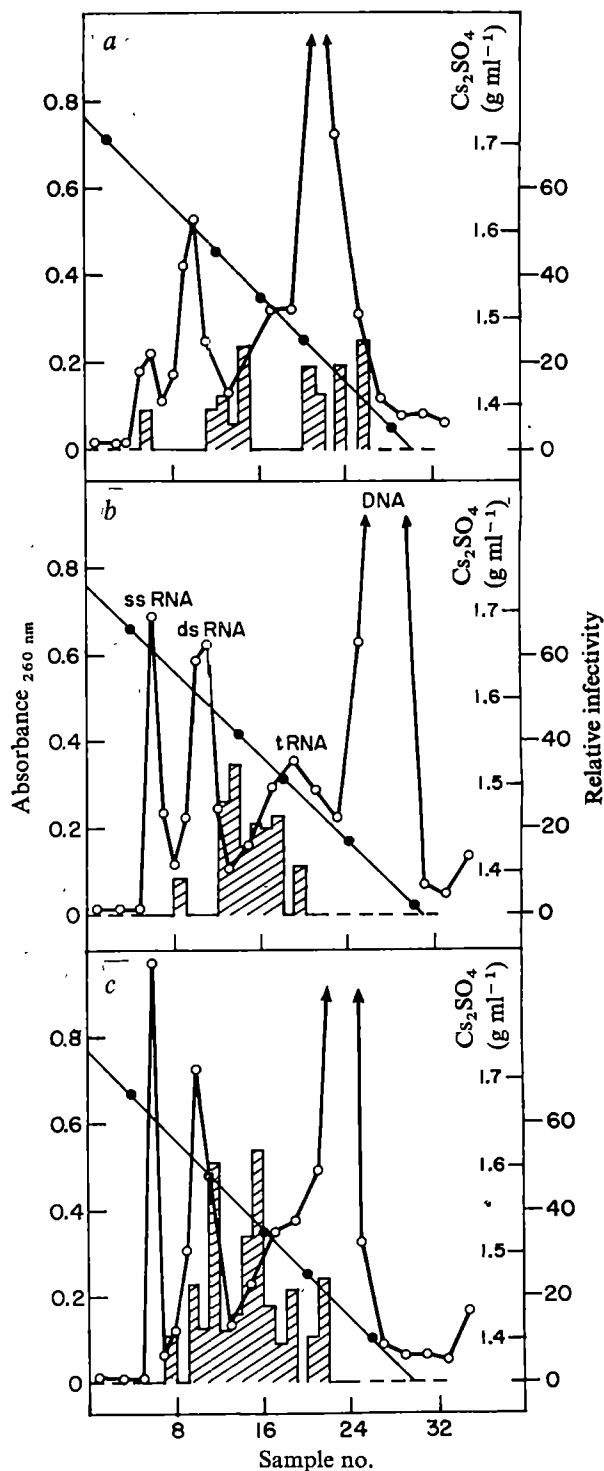Fig. 3 Sedimentation of DNA-rich preparation from exocortis-infected gynura (b) in 43% (w/w) Cs_2SO_4, after heating to 100 °C for 10 min and slow (a) or rapid cooling (c), in SSC buffer (0.1 M NaCl, 0.01 M sodium citrate, pH 7.0) centrifuged in the Beckman SW 50.1 rotor at 10 °C and 30,000 r.p.m. for 65 h. Samples were fractionated and analysed as in Fig. 2 and dialysed against TKM buffer before bioassay. Biological activity was determined by the appearance of stunting and epinasty symptoms on gynura over a 30-d period following inoculation by razor-slashing of the stems. The total number of 'infected-plant-days' on 3–4 gynura plants per sample comprised the relative infectivity[11] value (shaded areas). ○, A_{260nm}.

reflecting the unusual stability of the viroid RNA. The relatively normal distribution of relative infectivity in Cs_2SO_4 gradient observed in the control preparation (Fig. 3b), however, was

Table 1 Distribution of hybridisable nucleic acid in subcellular fractions from *Gynura aurantiaca* infected with CEV

Subcellular fraction	RNase resistant* [125]I (c.p.m.)	(%)	DNA (µg per 100 µl)	(%)	Relative infectivity Index	%
250g for 10 min	3,976	56	76	84	30	39
1,000g for 10 min	913	13	6.8	8	20	26
80,000g for 30 min	860	12	2.8	3	27	35
100,000g for 2 h	792	11	2.4	3	0	0
Supernatant	584	8	2.4	3	0	0

*35 µl of each DNA fraction used for hybridisation treatment. RNase-resistant ^{125}I was determined as described in Fig. 1.

altered in both the rapid and slow-cooled treatments to include a broader density range, encompassing the region of DNA. The distribution of the preparation reannealed in slow cooling conditions (Fig. 3a) is particularly striking, since a significant portion of the CEV–RNA now cosediments with the gynura DNA.

The CEV-specific DNA, concentrated in the 250-g pellet (Table 1) supports a nuclear phase in the synthesis of viroid RNA[12]. This is not to imply that the pathogenic RNA is totally restricted to the host nucleus[13]. Whether the DNA complementary to CEV–RNA is synthesised *de novo* by the action of an induced RNA-directed DNA polymerase, or exists in a limited number of copies and is amplified by normal DNA polymerase activity in the process of pathogenesis, remains to be determined. The mode of synthesis of CEV–RNA as well as the mechanism involved in the production of the pathological condition is critical to the description of the

J. S. Semancik

J. L. M. C. Geelen

Department of Plant Pathology and Cell Interaction Research Group, . *University of California,* *Riverside, California 92502*

Table 2 Hybridisation of ^{125}I-CEV–RNA to various DNA-rich preparations

DNA Source	RNase-resistant [125]I (c.p.m.)
Tobacco	70
Cowpea	0
Tomato	233
Gynura 5 µg	133
Gynura 10 µg	116
CEV-infected tomato	1,731
CEV-infected tomato+1.6 µg CEV–RNA	170
CEV-infected tomato+DNase*	350
CEV-infected gynura 5 µg	1,105
CEV-infected gynura 10 µg	1,465
CEV-infected gynura+1.6 µg CEV–RNA	153
CEV-infected gynura+DNase*	859

RNase-resistant ^{125}I was determined after hybridisation of 0.005 µg ^{125}I-CEV (50,000 c.p.m.) with 10 µg (except as indicated) of the DNA preparation as described in Fig. 1.
*Incubated 0.75 h at room temperature with 1 µg DNase I in a reaction volume of 40 µl.

[1] Semancik, J. S., and Weathers, L G., *Nature new Biol.*, 237, 242–244 (1972)
[2] Semancik, J S., Morris, T. J, Weathers, L. G., Rordorf, B. F., and Kearns, D. R., *Virology*, 63, 160–167 (1975).
[3] Semancik, J. S., *Virology*, 62, 288–291 (1974).
[4] Hall, T. C., Wepprich, R. K., Davies, J. W., Weathers, L. G., and Semancik, J. S , *Virology*, 61, 486–492 (1974).
[5] Diener, T. O., *Virology*, 45, 411–428 (1971).
[6] Diener, T. O., and Smith, D R., *Virology*, 63, 421–427 (1975).
[7] Geelen, J. L. M. C , Weathers, L. G., and Semancik, J S., *Virology* (in the press)
[8] Friedrick, R., and Feix, G., *Analyt. Biochem.*, 50, 467–476 (1972).
[9] Prensky, W , Steffensen, D M., and Hughes, W. L., *Proc. natn. Acad. Sci. U.S A.*, 70, 1860–1864 (1973)
[10] Bishop, D. H. L., Claybrook, J. R., and Spiegelman, S., *J. molec. Biol.*, 26, 373–387 (1967).
[11] Semancik, J S , and Weathers, L. G , *Virology*, 47, 456–466 (1972)
[12] Takahashi, T , and Diener, T. O., *Virology*, 64, 106–114 (1975)
[13] Semancik, J. S , Tsuruda, D., Zaner, L M., Geelen, J. L. M. C., and Weathers, L. G., *Virology* (in the press)

unique class of pathogenic RNA, the viroids. The CEV–RNA reflects neither the structural[2] nor the biological properties[3,4] of most plant viral nucleic acids. This report further supports a unique role for the viroid RNA as a regulator of the expression of the normal host genome resulting in the production of a 'disease-like' symptom. The implied participation of a DNA intermediate reported here as well as the observed requirement for cell division in the synthesis of CEV–RNA (J.S.S., unpublished) further supports the intimate interaction between the viroid RNA and host cell, resulting in a condition analogous to cell transformation.

Presence of sequences hybridisable to dsRNA in cytoplasmic mRNA molecules

IN recent years, a precursor–product relationship between heterogeneous nuclear RNA (HnRNA) and cytoplasmic messenger RNA (mRNA) has been investigated intensively[1,2]. The HnRNA was shown to have a double-stranded hairpin structure within its long molecules[3–5]. This particular region has been isolated as a double-stranded RNA (dsRNA) after treatment with ribonucleases, followed by purification on cellulose[5] or hydroxyapatite[3,4]. The HnRNA of higher organisms is degraded largely in the nucleus and, at most, 10% of the nuclear content

is transported to the cytoplasm[2]. It remains unknown whether the double-stranded structures in the HnRNA molecules are totally degraded in the nucleus or whether sequences involved in this structure are preserved in part or *in toto* during nuclear processing and possibly become parts of cytoplasmic mRNA.

We investigated the possibility that sequences from double-stranded structures are present in cytoplasmic mRNA molecules and present data showing that there are sequences hybridisable to denatured dsRNA in mRNA molecules and that these sequences are 6.1% of the total mRNA. We also tried to investigate the relative position of these sequences in mRNA molecules using 5'-exonuclease and polynucleotide phosphorylase.

The experiment consists of the molecular hybridisation between labelled mRNA and unlabelled dsRNA, both prepared from rat livers. To estimate the percentage hybridisation of mRNA to dsRNA, purified mRNA was annealed to dsRNA at varying concentrations, but at a fixed input ratio (dsRNA: mRNA, w/w) of 1:1. This input ratio was chosen since hybrids formed at low input ratio, that is, 1:1, exhibited a sharp melting profile with a T_m only 6 °C below that of the original dsRNA (ref. 6), indicating a reasonably good fidelity of base pairing.

mRNA hybridised to dsRNA on annealing (Fig. 1) and the hybridisation reaches a plateau at a value of approximately 30 mg ml^{-1} h^{-1}. Since contamination of nuclear RNA, including dsRNA, in the mRNA preparation was extremely low[6], this observation did not result from the hybridisation of labelled dsRNA (contaminating the labelled mRNA preparation) to the added dsRNA, but certainly represents the hybrid formation between labelled mRNA and dsRNA added. The hybridisation curve shows that dsRNA hybridised to 6.1% of mRNA added. Assuming that the average value for the length of an mRNA molecule is approximately 1,000 nucleotides[11], then the total length of the sequences involved would be approximately 60 nucleotides. This is within the size range of purified rat liver dsRNA, as estimated by electrophoresis on 10% acrylamide gels[6] and by electron microscopy according to the aqueous technique of David *et al.*[12]. The purified dsRNA showed a broad size distribution.

This observation is of particular importance with respect to the hypothesis that HnRNA is a precursor of cytoplasmic mRNA (ref. 13). It has been demonstrated that all dsRNA is of nuclear origin and is not derived from ribosomal, preribosomal and transfer RNAs (refs 3—5). In particular, dsRNA obtained from high molecular weight nuclear RNA is in the form of a hairpin structure in the HnRNA molecule with a linking nucleotide sequence at the top of the hairpin (refs 3, 4 and R. P. Monckton and H. N., unpublished). No definite double-stranded structure has been detected in the cytoplasm[5] or in the cytoplasmic mRNA[3,6]. Electron microscopic observations of isolated mRNA molecules did not reveal any double-stranded structure either. Although a secondary structure for particular species of eukaryotic mRNA has been suggested after examining amino acid sequences[14,15] and performing hyperchromicity experiments[16], no conclusive evidence has yet been reported. In addition, denatured dsRNA prepared from nuclear RNA and completely free of cytoplasmic mRNA also hybridised to mRNA. Therefore, the presence of sequences in the mRNA molecule hybridisable to dsRNA indicates that, if no post-transcriptional addition of these sequences to mRNA occurs, mRNA is most likely to be derived from sequences contained in HnRNA. The absence of actual double-stranded structures in mRNA is presumably because only a portion of the sequences necessary for double-stranded hairpin structuring is retained during the processing of HnRNA to mRNA. There is considerable evidence supporting the hypothesis of a precursor-product relationship of HnRNA and mRNA. What doubt there is arises mainly from the fact that most of the experiments did not completely eliminate the possibility of the presence of cytoplasmic mRNA contaminating the HnRNA preparation[17,18]. Our present observations support the possible precursor-product relationship between HnRNA and mRNA

without any doubt of contamination by mRNA, since purified dsRNA is derived from HnRNA and is free of cytoplasmic mRNA (ref. 5).

We then investigated whether sequences hybridisable to dsRNA are located at or near the 5'- and (or) 3'-end of the cytoplasmic mRNA molecule. The hybrids between dsRNA
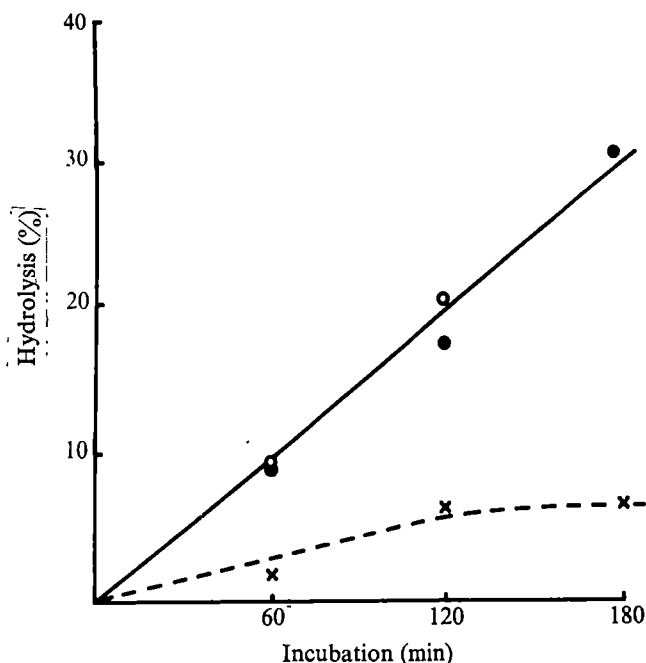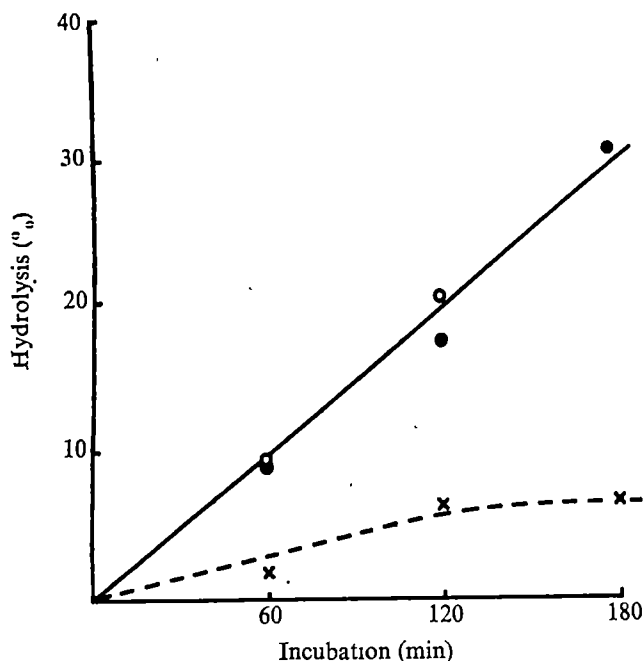

Fig. 1 Hybridisation of ^{3}H-labelled mRNA to unlabelled dsRNA. For preparation of ^{3}H-labelled mRNA, ^{3}H-orotic acid (300 μCi) was injected into rat portal vein 4 h before decapitation. Polysomes were obtained from labelled livers according to the method of Schreier and Staehelin[7] and then polysomal RNA was extracted by treatment with chloroform–phenol[8]. mRNA was isolated from polysomal RNA by sucrose density gradient centrifugation as described previously[6]. Our mRNA preparation has already been characterised and exhibited messenger activity in a cell-free protein-synthesising system[6]. dsRNA was prepared and purified from rat livers as previously[5]. The preparation procedure involved extensive treatment of whole cell or nuclear RNA with DNase, Pronase and RNases (A and T₁). The crude preparation of dsRNA was purified on a cellulose column. The dsRNA preparation was completely free of DNA[5]. All hybridisation reactions were carried out at 72 °C for 24 h in hybridisation solution (10 mM phosphate buffer, pH 7.6, 0.3 M NaCl) by a modification of Schonberg *et al*[9] and Shimotohno and Miura[10]. Larger values of mg ml^{-1} h^{-1} were obtained by increasing the concentration of RNA and not by lengthening the reaction time. After annealing, the reaction mixture was diluted to a final volume of 0.2 ml with hybridisation solution and subjected to treatment with 10 μg ml^{-1} of RNase A and 0.5 μg ml^{-1} of RNase T₁ at 37 °C for 30 min. It was shown that the RNase-resistance observed after annealing certainly results from hybrid formation[6]. The dsRNA–mRNA hybrids formed were precipitated in the presence of carrier RNA with trichloroacetic acid, collected on glass fibre filters and the radioactivity determined[6]. Since 0.6% of mRNA became RNase resistant after annealing without dsRNA, this 'blank' value was subtracted from the measured values. The RNase-resistant materials obtained after annealing without dsRNA did not behave as typical double-stranded structures, since this resistance to RNases was clearly not lost after thermal denaturation.

and labelled mRNA were treated with either spleen phosphodiesterase (with or without added phosphomonoesterase) or polynucleotide phosphorylase. The secondary structure of polynucleotides is insensitive to these enzymes, which attack

only single-stranded polynucleotides from the 5'-end (spleen phosphodiesterase[19]) or 3'-end (polynucleotide phosphorylase[20]) of the molecule.

Treatment of ^{3}H-labelled heat-denatured hybrids or cytoplasmic mRNA alone with spleen phosphodiesterase in the conditions described in Fig. 2 released radioactivity almost linearly with incubation time (Fig. 2). Hydrolysis of the intact hybrids took place at a much slower rate in the same conditions during incubation for 60–180 min. Since commercial preparations of spleen phosphodiesterase contain contaminating

between intact and denatured hybrids suggests that sequences hybridisable to dsRNA do not occur in clusters along the entire molecule, as may be the case in a special class of RNA which would be a small proportion of the mRNA population, and that most of the mRNA molecules possess sequences hybridisable to dsRNA. This is indeed the case, since hybrids were formed between dsRNA and 98% of the ^{3}H-labelled mRNA population as evidenced by the retention of ribonuclease-untreated hybrids on a hydroxyapatite column. The mRNA molecule seems likely to be composed partly of sequences

Fig. 2 Hydrolysis of dsRNA–mRNA hybrids by spleen phosphodiesterase. The hybrids formed with denatured dsRNA and ^{3}H-labelled mRNA (approximately 1,000–1,500 c.p.m.) at an input ratio of 1:1 at a value of 10–15 mg ml^{-1} h^{-1} were treated at 37 °C with 250 μg ml^{-1} of spleen phosphodiesterase (Worthington Biochem. Corp.) in hydrolysis solution (50 mM Tris-HCl, pH 8.0, 10 mM MgCl$_2$, 0.3 M NaCl) based on the conditions used for reducing the activity of contaminating endonucleases[21]. Phosphodiesterase activity still remains at high pH (ref. 22). The hybrid preparations used here were not purified after annealing and so contained some residual unhybridised mRNA. After incubation, the samples were chilled in ice and trichloroacetic acid was added to terminate the reaction. The radioactivity of the acid-insoluble residue was measured as described in Fig. 1. Heat-denatured (100 °C for 10 min, followed by immediate cooling in ice) hybrids (●) were used as a control for comparison of the susceptibility of intact hybrids (×). Hydrolysis of mRNA alone (○) was also monitored. This occurred in exactly the same fashion as that of denatured hybrids.

Fig. 3 Phosphorolysis of dsRNA–mRNA hybrids by polynucleotide phosphorylase. The hybrids formed in the conditions described in Fig. 2 were treated at 37 °C with 125 units ml^{-1} of polynucleotide phosphorylase (*Micrococcus lysodeikticus*, Sigma Chemical Co.) in the presence of 1 M NaCl under the conditions described by Soreq *et al.*[24]. After incubation, the samples were treated as described in Fig. 2. ○, Heat-denatured; ×, intact hybrids.

phosphomonoesterase activities[23], addition of phosphomonoesterase (0.45–1.7×10^{-3} units per μg RNA, Sigma Chemical Co.) to the incubation mixture resulted in only a 10–15% increase in hydrolysis. The low susceptibility of the intact hybrids to spleen phosphodiesterase suggests that there are almost no long single-stranded tails on the 5'-end of the hybrids. This suggests the presence of sequences hybridisable to dsRNA at or near the 5'-end of mRNA molecules. The possibility that mRNAs contain these sequences also at or near the 3'-end cannot, however, be excluded. In fact, intact hybrids were also less susceptible than denatured hybrids to degradation from the 3'-end by polynucleotide phosphorylase (Fig. 3).

The remarkable difference in susceptibility to these enzymes

hybridisable to dsRNA, partly of a unique sequence coding for polypeptide and partly of a poly(A) segment. The results obtained here reveal nothing of the presence or absence of sequences hybridisable to dsRNA in the internal positions of the mRNA molecule; however, since a major portion of the mRNA molecule is the unique sequence coding for polypeptide, sequences hybridisable to dsRNA are unlikely to be in the middle of the molecule. Such sequences would seem to be at or near the 5'-end and also between the unique sequence and the poly(A) segment at the 3'-end. There is evidence suggesting the presence of these sequences at or near the 5'- and 3'-ends of the mRNA molecules. First, sequences transcribed from repetitious DNA sequences, from which dsRNA is transcribed, have been detected at or near the potential 5'-end of the mRNA portion of HnRNA molecules[25]. Second, repetitious sequences were actually found at the 5'-end of *Xenopus* embryo mRNA[26], and both at the 5'- and 3'-ends of slime mould mRNA and nuclear RNA (ref. 27).

Taking all these facts into account, it is suggested that with the exception of some mRNAs, cytoplasmic mRNA is at least

composed of a sequence corresponding to a portion of one strand of dsRNA both at, or near, the 5'-end and probably between the unique sequence coding for polypeptide and the poly(A) segment at the 3'-end. The existence of sequences hybridisable to dsRNA is consistent with the hypothesis that the dsRNA hairpin structure of the HnRNA molecule is a cleavage point which may be specifically recognised by a nuclear processing enzyme. This implies that the poly(A) segment linked to the unprocessed HnRNA molecule would be detached during the maturation process and a new poly(A) segment would be added to the 3'-end after completion of the processing. Recent experiments have revealed that much of the poly(A) segment attached to nuclear RNA decays in the nucleus and is not conserved[2,28]. None of the experiments carried out so far on polyadenylation excludes the possibility that poly(A) may be added to new 3'-ends generated by the processing of HnRNA as well as to the 3'-end of the unprocessed HnRNA molecule[2]. Moreover, it seems likely that addition of a poly(A) segment takes place both before and after nuclear processing of HnRNA molecules[29].

H. NAORA
J. M. WHITELAM

Molecular Biology Unit,
Research School of Biological Sciences,
Australian National University,
Canberra, ACT 2601, Australia

1 Brawerman, G., *A. Rev Biochem.*, 43, 621-642 (1974)
2 Lewin, B., *Cell*, 4, 11-20 (1975)
3 Jelinek, W, and Darnell, J. E., *Proc. natn. Acad Sci. U.S A.*, 69, 2537-2541 (1972).
4 Ryskov, A. P, Saunders, G F, Farashyan, V. R., and Georgiev, G P, *Biochim. biophys Acta*, 312, 152-164 (1973).
5 Monckton, R. P., and Naora, H., *Biochim. biophys. Acta*, 335, 139-154 (1974)
6 Naora, H., Whitelam, J. M., Monckton, R. P, and Wettenhall, R E. H., in *The Eukaryote Chromosome* (edit. by Peacock, W. J., and Brock, R D.), (Australian National University Press, 1975).
7 Schreier, M. H., and Staehelin, T., *J. molec Biol*, 73, 329-349 (1973).
8 Perry, R P, La Torre, J, Kelley, D. E, and Greenberg, J. R, *Biochim biophys Acta*, 262, 220-226 (1972).
9 Schonberg, M., Silverstein, S C, Levin, D. H., and Acs, G, *Proc. natn Acad Sci. U.S A.*, 68, 505-509 (1971).
10 Shimotohno, K., and Miura, K., *Virology*, 53, 283-286 (1973).
11 Davidson, E D, and Britten, R J, *Q. Rev Biol*, 40, 565-613 (1973).
12 Davis, R. W, Simon, M., and Davidson, N., in *Methods in Enzymology*, XXI (edit. by Grossman, L, and Moldave, L K), 413-428 (Academic, New York and London, 1971)
13 Darnell, J E., Jelinek, W. R, and Molloy, G R, *Science*, 181, 1215-1221 (1973).
14 White, H. B., III, Laux, B E, and Dennis, D, *Science*, 175, 1264-1266 (1972)
15 Riley, W. T, *J. theor Biol*, 40, 285-300 (1973)
16 Williamson, R., Morrison, M, Lanyon, G, Eason, R., and Paul, J, *Biochemistry*, 16, 3014-3021 (1971).
17 Busch, H, in *The Molecular Biology of Cancer* (edit. by Busch, H), 187-239 (Academic, New York, 1974)
18 McKnight, G S, and Schimke, R. T., *Proc. natn Acad Sci U S A*, 71, 4327-4331 (1974)
19 Bernardi, A, and Bernardi, G, in *The Enzymes*, IV 3rd ed (edit by Boyer, P. D.), 329-336 (Academic, New York and London, 1971)
20 Grunberg-Manago, M, *Prog Nucl. Acid Res.*, 1, 93-133 (1963)
21 Stulberg, M P, and Isham, K. R, in *Methods in Enzymology*, XXIV (edit by Grossman, L, and Moldave, L K), 477-482 (Academic, New York and London, 1974)
22 Hilmoe, R. J, *J biol Chem*, 235, 2117-2121 (1960)
23 Whitecome, P, Fry, K., and Jalser, W., in *Methods in Enzymology*, XXIX (edit by Grossman, L and Moldave, L K), 295-321 (Academic, New York and London, 1974)
24 Soreq, H, Nudel, U, Salomon, R, Revel, M, and Littauer, U. Z, *J molec Biol*, 88, 233-245 (1974)
25 Molloy, G. R, Jelinek, W, Salditt, M., and Darnell, J E, *Cell*, 1, 43-53 (1974)
26 Dina, D., Meza, I, and Crippa, M, *Nature*, 248, 486-490 (1974)
27 Firtel, R. A, and Lodish, H F, *J molec. Biol*, 79, 295-314 (1973)
28 Perry, R P, Kelley, D E, and La Torre, J, *J molec. Biol*, 82, 315-332 (1974).
29 Derman, E, and Darnell, J E, *Cell*, 3, 255-264 (1974)

Identification of the high and low affinity CO₂-binding sites of human haemoglobin

THE unloading of oxygen in the tissues is facilitated by lowering the oxygen affinity of haemoglobin (Hb) by both CO_2 and diphosphoglycerate (DPG)[1]. This occurs because CO_2 and DPG are oxygen-linked, that is, they are bound more firmly to deoxyhaemoglobin than to oxyhaemoglobin[2,3]. The binding of

CO_2 to Hb takes place by a reaction of CO_2 with the α-amino groups to form carbamino compounds[4,5], and binding curves can be measured by rather laborious methods[4,6]. Perrella *et al.*[7] have modified these methods so that much smaller amounts of Hb, such as the specifically carbamylated Hbs[8], can be used. In this method, Hb equilibrated with CO_2 is rapidly taken to pH 11 to stabilise the carbamino CO_2 and BioRad AG 1×8 resin added to remove carbonate and bicarbonate ions. The carbamino CO_2 is displaced from the Hb by acidification and measured in a Van Slyke apparatus. DPG-free human deoxyhaemoglobin gave diphasic CO_2 binding curves[7] showing that the affinities of the α and β chain α-amino groups for CO_2 are different, but it was impossible to establish which group had the higher affinity.

We have used the specifically carbamylated Hbs, $\alpha_2^C\beta_2$ and $\alpha_2\beta_2^C$ where c denotes specific reaction of the α-amino group with cyanate[8], to identify the low and high affinity sites for CO_2 in Hb.

As a control experiment we first measured the CO_2 bound to $\alpha_2^C\beta_2^C$; this amount was very small but not negligible and was about 10% of that bound to normal Hb in the same
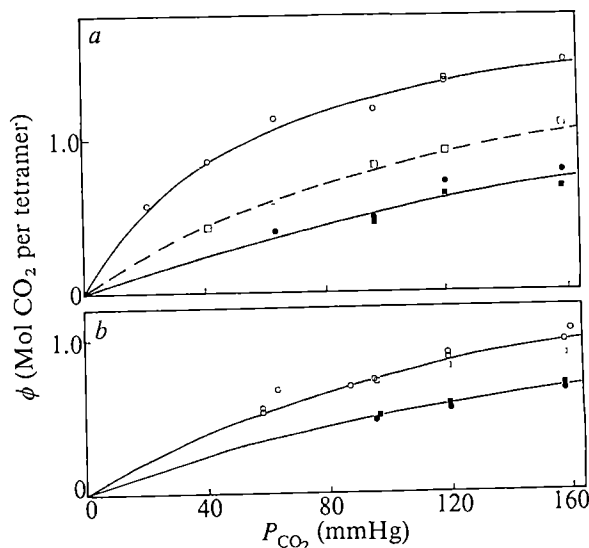

Fig. 1 CO_2 bound by $\alpha_2^C\beta_2$ (*a*) and $\alpha_2\beta_2^C$ (*b*) at pH 7.4, 37 °C. Haemoglobin concentration 3.5 mM in tetramer, ionic strength 0.18. O, Deoxy; □, deoxy + 2 mol DPG per tetramer; ●, CO, ■, CO + 2 mol DPG per tetramer. The data were corrected for a 5% contamination with normal Hb. The P_{CO_2} can be converted to mM CO_2 by multiplying the value of mmHg by 0.031. The solid lines through the points are the calculated curves with values of the pH-dependent association constants λ of λ_β = 579 M⁻¹ and λ_β^{CO} = 120 M⁻¹ for deoxy and CO $\alpha_2^C\beta_2$ and λ_α = 190 M⁻¹ and λ_α^{CO} = 100 M⁻¹ for deoxy and CO $\alpha_2\beta_2^C$ The broken line is the curve calculated from equation (1) (using a program written by Dr A. P. Minton) with K_p = 5 × 10³ M⁻¹, K_p' = 1,700 M⁻¹, and K_p'' = 500 M⁻¹.

conditions. This small amount of oxygen-linked CO_2 could be bound to a site different from the α-amino groups or could be the result of about 10% contaminating normal Hb. The CO_2 bound to $\alpha_2^C\beta_2$ and $\alpha_2\beta_2^C$ is shown in Fig. 1.

In the DPG-free deoxy forms $\alpha_2^C\beta_2$ binds more CO_2 than $\alpha_2\beta_2^C$, while in the CO forms both bind CO_2 equally. The calculated pH-dependent association constants (Fig. 1) show that in the deoxy form the β chain α-amino group has about a threefold higher affinity for CO_2 than the α chain α-amino group. This is about the same difference found between the

two unidentified binding sites in normal human deoxyhaemo-globin[7], and is in agreement with the effect of CO_2 on the oxygen affinity of $\alpha_2^C\beta_2$ and $\alpha_2\beta_2^C$ (ref. 8).

Figure 2 shows that the addition of the CO_2 bound by $\alpha_2^C\beta_2$ and $\alpha_2\beta_2^C$ is nearly the same as the total CO_2 bound by normal Hb. This indicates that the free α-amino groups in $\alpha_2^C\beta_2$ and $\alpha_2\beta_2^C$ bind the same amount of CO_2 as the same groups in normal Hb and these derivatives can thus be considered good model compounds for the study of normal Hb.

DPG does not affect the CO_2 binding of deoxy $\alpha_2\beta_2^C$ or the CO forms of $\alpha_2\beta_2^C$ and $\alpha_2^C\beta_2$, but reduces the CO_2 binding of deoxy $\alpha_2^C\beta_2$ by about 30% (Fig. 1), in agreement with the fact that DPG interacts with the β chain α-amino group of deoxy-
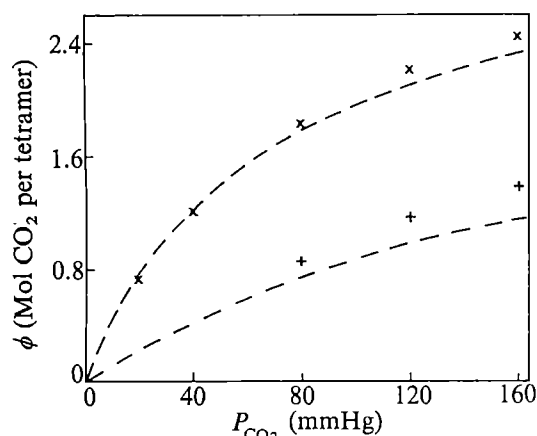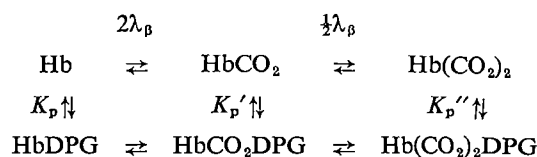

Fig. 2 CO_2 bound by normal deoxyhaemoglobin (– – – –) and CO-haemoglobin (– . – . – . –) in the same conditions as Fig. 1, taken from ref. 7. The symbols are the sum of the two sets of CO_2 binding data for deoxy (×) and CO (+) $\alpha_2^C\beta_2^C$ and $\alpha_2\beta_2$ taken from Fig. 1.

haemoglobin[9]. The total CO_2 bound to $\alpha_2^C\beta_2$ and $\alpha_2\beta_2^C$ in the presence of DPG equals that bound to normal deoxyhaemo-globin in the same conditions. Thus at 42 mmHg P_{CO_2}, deoxy $\alpha_2^C\beta_2$ and $\alpha_2\beta_2^C$ bind, in total, 0.64 mol CO_2 per tetramer (Fig. 1) compared with the 0.68 mol CO_2 per tetramer found, in this study, bound to normal deoxyhaemoglobin. A similar reduction in CO_2 binding by DPG or ATP was found by Brenna *et al.*[1].

X-ray crystallography shows that binding of two CO_2 molecules to the β chain α-amino groups[10] would decrease the binding of DPG, but it is very difficult to make a quantitative estimate from these data. The CO_2 binding data of deoxy $\alpha_2^C\beta_2$ in the presence of DPG can be used to estimate values of K_p' and K_p'' (the DPG association constants when one or two CO_2 molecules are bound) provided that the value of K_p (the DPG association constant of $\alpha_2^C\beta_2$ in the absence of DPG) is known. We have used $\alpha_2^C\beta_2$ rather than normal Hb because complications caused by CO_2 binding to the α chain α-amino group are avoided. The K_p values measured in conditions similar to ours (that is, concentrated deoxyhaemoglobin at 37 °C) are 10^4 M^{-1} (ref. 11) and 5×10^3 M^{-1} (ref. 12) at pH 7.2 and ionic strength 0.12 and 0.15, respectively. Our conditions of pH 7.4 and ionic strength 0.18 should decrease these values only slightly[3]. The K_p for $\alpha_2^C\beta_2$ is likely to be the same as for deoxyhaemoglobin because DPG causes the same change in log P_{50} as in normal Hb[8]. This shows that the difference between the DPG association constants of the oxy- and deoxy-forms of $\alpha_2^C\beta_2$ are the same as for normal Hb, and it is likely that their absolute values are the same.

If DPG binds to only one site in deoxyhaemoglobin and CO_2 and DPG can simultaneously bind at this site, then the data can be analysed by the following scheme

$$
\begin{array}{ccccc}
& 2\lambda_\beta & & \tfrac{1}{2}\lambda_\beta & \\
\text{Hb} & \rightleftarrows & \text{HbCO}_2 & \rightleftarrows & \text{Hb(CO}_2)_2 \\
K_p \updownarrow & & K_p' \updownarrow & & K_p'' \updownarrow \\
\text{HbDPG} & \rightleftarrows & \text{HbCO}_2\text{DPG} & \rightleftarrows & \text{Hb(CO}_2)_2\text{DPG}
\end{array}
$$

where Hb represents $\alpha_2^C\beta_2$, λ_β is the association constant for CO_2 binding to the β chain α-amino group[7], K_p is the association constant for DPG binding to deoxy $\alpha_2^C\beta_2$ in the absence of CO_2, and K_p' and K_p'' the DPG association constants when one CO_2 or two CO_2 molecules are bound. The number of mol per tetramer of CO_2 (φ) bound to the β chain α-amino group in the presence of DPG, is

$$
\varphi = \frac{2\lambda_\beta[CO_2](1+\lambda_\beta[CO_2]+K_p'[DPG]+\lambda[CO_2]K_p''[DPG])}{(1+\lambda_\beta[CO_2])^2+K_p[DPG]+2\lambda_\beta[CO_2]K_p'[DPG]+ \\ +\lambda_\beta{}^2[CO_2]^2K_p''[DPG]} \quad (1)
$$

The value of K_p in equation (1) can be determined when the ratio K_p/K_p'' is known. When K_p/K_p'' is less than 10, then large values of $K_p(>2\times10^4$ $M^{-1})$ will fit the data in Fig. 1. As K_p/K_p'' is progressively increased, then K_p must be decreased to fit the data. Thus, if $K_p/K_p''>100$ then K_p must be less than 10^3 M^{-1}, a value clearly too small in respect to the directly-measured values of K_p (refs 11, 12). Figure 1 shows the fit obtained with $K_p/K_p'' = 10$ and K_p set at 5×10^3 M^{-1} to comply with the directly-measured values[11,12]. This low ratio of K_p/K_p'' shows that substantial populations of the species HbCO$_2$DPG and Hb(CO$_2$)$_2$DPG must exist.

Brenna *et al.*[1] attempted to identify the high affinity CO_2 binding site from the CO_2 binding data of normal deoxy-haemoglobin in the presence of ATP or DPG. They used $K_p = 10^4$ M^{-1} but erroneously assumed, in absence of crystallo-graphic data, K_p' and K_p'' to be zero. When these values of K_p, K_p', and K_p'' are used in equation (1) a low value of λ_β is calculated, suggesting that the high affinity CO_2 binding site in deoxyhaemoglobin is the α chain α-amino group. Clearly our finding of substantial populations of HbCO$_2$DPG and Hb(CO$_2$)$_2$DPG species invalidates the simplified analysis of Brenna *et al.*[1].

Our data are partly in agreement with the crystallographic work of Arnone[10] who found clear evidence of CO_2 binding to the β chain α-amino group but no CO_2 on the α chain α-amino groups and instead CO_2 bound to the interior of the protein near the β chain haem group. It is not clear whether this CO_2 would still be bound in solution in the red cell or whether it would be oxygen linked. The failure to observe CO_2 binding to the α chain α-amino group is surprising because $\alpha_2\beta_2^C$ clearly binds CO_2, which is practically abolished when its free α-amino group is blocked in $\alpha_2^C\beta_2^C$.

We thank Drs A. Szabo and A. P. Minton for discussion, and Mr M. Chimisso for experimental help. This research was supported by the Consiglio Nazionale delle Ricerche, Rome.

M. Perrella

Cattedra di Enzimologia,
University of Milan

J. V. KILMARTIN
J. FOGG

MRC Laboratory of Molecular Biology,
Hills Road, Cambridge CB2 2QH, UK

L. ROSSI-BERNARDI

Cattedra di Enzimologia,
University of Milan, 2 Via G. Celoria,
20133 Milan, Italy

1 Brenna, O., et al., in Advances in Experimental Medicine and Biology (edit by Brewer, G. J.), 19–37 (Plenum, New York, 1972).
2 Rossi-Bernardi, L., and Roughton, F. J. W., J. Physiol., Lond., 189, 1–29 (1967)
3 Benesch, R. E., Benesch, R., and Yu, C. I., Biochemistry, 8, 2567–2571 (1969).
4 Ferguson, J. K. W., and Roughton, F. J. W., J. Physiol., Lond., 83, 87–102 (1934).
5 Kilmartin, J. V., and Rossi-Bernardi, L., Nature, 222, 1243–1246 (1969)
6 Perrella, M., Rossi-Bernardi, L., and Roughton, F. J. W., in A. Benzon Symp. IV, Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status (edit. by Rorth, M., and Astrup, P.), 177–203 (Munksgaard, Kobenhavn, 1971).
7 Perrella, M., Bresciani, D., and Rossi-Bernardi, L., J. biol. Chem. 250, 5413–5418 (1975).
8 Kilmartin, J. V., Fogg, J., Luzzana, M., and Rossi-Bernardi, L., J. biol. Chem., 248, 7039–7043 (1973).
9 Arnone, A., Nature, 237, 146–149 (1972).
10 Arnone, A., Nature, 247, 143–145 (1974).
11 Hamasaki, N., and Rose, Z. B., J. biol. Chem., 249, 7896–7901 (1974)
12 Berger, J., Janig, G., Gerber, G., Ruckpaul, K., and Rapoport, S. M., Eur. J. Biochem., 38, 553–562 (1973).

Spectral changes and allosteric transition in trout haemoglobin

PREVIOUS results have shown that two of the haemoglobin components from trout blood, Hb trout I and Hb trout IV, have different functional properties, which may be correlated directly with their physiological role (see ref. 1 for review). Thus, Hb trout I, which displays cooperative ligand binding at all pH values, completely lacks heterotropic interactions; on the other hand, these interactions are clearly evident in Hb trout IV, for which lowering of pH, or addition of organic phosphates, produces a large decrease in oxygen affinity and in cooperativity (n drops from 2.3 at pH 8 to ~ 1 at pH 6)[2,3].

Although the molecular interpretation of the Root effect in Hb trout IV is certainly complex, it is probably related to the stabilisation, induced by protons, of a "low affinity" conformational state of the molecule, according to a simplified "two states" model[1]. Such evidence has been provided through EPR experiments on the NO-derivative of Hb trout IV (ref. 4). Here we report further evidence that protons and inositol hexaphosphate (IHP) can induce characteristic shifts in the absorption spectrum of the carbon monoxide derivative of Hb trout IV.

Haemoglobin components from trout blood (from Salmo irideus) were purified as described previously[2]. All reagents were analytical grade and were used without further purification. Absolute and difference spectra were recorded with a Cary 14 spectrophotometer thermostated at 20 °C.

The Soret absorption band of Hb trout IV fully saturated with carbon monoxide is redshifted by about 1 nm going from pH 8 to 6, as shown by the difference spectra reported in Fig. 1a. Control experiments have shown clearly that the observed difference spectra cannot be attributed to the presence of methaemoglobin contaminations, or to changes in the saturation with CO. In addition it should be emphasised that no pH-dependent difference spectra of comparable magnitude have been detected for the deoxygenated derivative of Hb trout IV, or for Hb trout I both in the carbomonoxy- and in the deoxyform.

Figure 1b points out that somewhat similar difference spectra are obtained, at constant pH, by addition of IHP. The effect of the organic phosphate seems, however, to be more complex, as different isosbestic points were observed at low and high pH
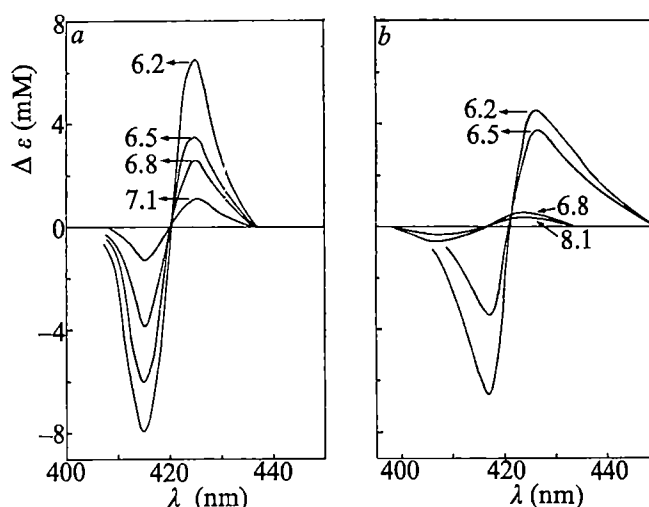

Fig. 1 Effect of pH and IHP on the spectrum of carbomonoxyhaemoglobin trout IV in 0.05 M bis-Tris at 20 °C. a, Difference absorption spectra at various pH, as indicated (reference cell, pH 8.1). b, Difference absorption spectra $\pm 10^{-3}$ M IHP at constant pH, as indicated. Protein concentration 6 μm (in haem).

values. The results obtained at constant proton activity indicate, in agreement with the functional data[5], an additional stabilising effect of IHP on the "low affinity" form of liganded Hb trout IV. The titration of Hb trout IV–CO with IHP is shown in Fig. 2. It corresponds to a simple hyperbola, with a value for the overall affinity constant (K) of 1.4×10^4 M^{-1} at pH 6.2 and
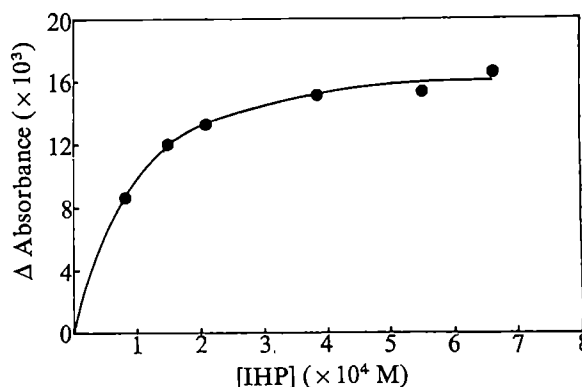

Fig. 2 Binding curve of IHP to Hb trout IV–CO in 0.05 M bis-Tris at 20 °C. Δ Absorbance are the amplitudes of the difference spectra obtained in the presence of increasing amounts of IHP.

20 °C. This value is considerably smaller than that reported for oxygenated human haemoglobin ($K \approx 10^6$ M^{-1} at pH 7.0 and room temperature)[6]. The difference, although puzzling, is not inconsistent with the known changes in some of the amino acid residues involved in the interactions with IHP within the central cavity of the tetramer[7].

The existence of isosbestic points, at 420 and 437 nm, in the pH-dependent difference spectra, is in agreement with the presence of only two spectral species in pH-dependent equilibrium. The experimental data in bis-Tris (0.05 M) can be fitted with a simple titration curve, with pK 6.5 (Fig. 3), suggesting that only one type of ionisable group is involved in the Root effect. In the presence of IHP (10^{-3} M) a similar effect is observed
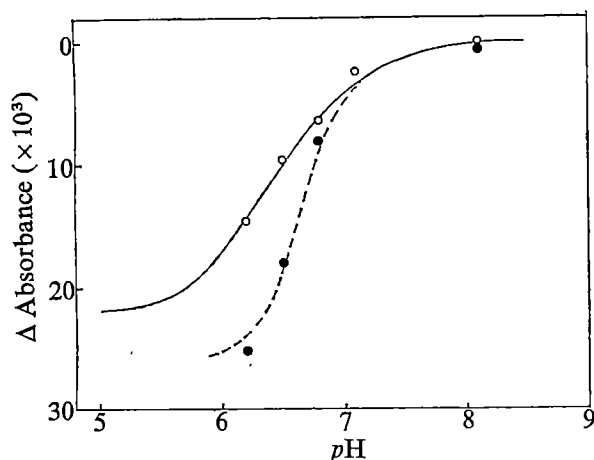

Fig. 3 pH-induced transition in Hb trout IV–CO in the absence (O) and in the presence (●) of 10^{-3} M IHP (solvent 0.05 M bis-Tris buffer) at 20 °C; Δ Absorbance as in Fig. 2.

(Fig. 3); the higher pK of the transition is consistent with the cumulative effect that both protons and the organic phosphate exert on the stabilisation of the "low affinity" form. It cannot be excluded, however, that in the presence and absence of IHP the proton induced transition may occur among different states. The greater steepness of the transition observed in IHP is probably related to the fact that Hb trout IV is not fully saturated with the phosphate at all pH values.

It is difficult to interpret the redshift of the maximum of absorption of the CO derivative of Hb trout IV, induced by lowering pH. For instance, it is not possible to correlate unequivocally the position of the peak to the strength of the bond between the iron and the ligand, although obviously the observed changes must reflect a perturbation in the distribution of electrons in the porphyrin–metal–ligand complex. The observed redshift may suggest a weakening of the π-bonding and an increase in the iron–ligand bond length correlated to a movement of the proximal imidazole relative to the metal (R.J.P. Williams, personal communication). Apart from detailed interpretations of the observed effect, it is of interest to correlate the spectral change with other properties of Hb trout IV, which are also pH dependent and occur over the same pH region. First, the dramatic change in functional properties and in particular the well known drop in ligand affinity which is experienced between pH 8 and 5. It seems especially relevant to remark that such a drop in affinity, common to fish haemoglobins, can be largely attributed to a great increase in the dissociation velocity constant of the ligand[8], which in itself is an indication of a destabilisation of the ligand–protein complex. Second, the fact that pH-dependent ultraviolet difference spectra of Hb trout IV saturated with CO have been observed (M. F. Perutz, personal communication and unpublished data from this laboratory). These difference spectra show a characteristic "fine structure", centred between 280 and 290 nm, which in the case of human haemoglobin has been directly correlated

with the ligand-induced change in the quaternary structure of haemoglobin, and thus should reflect the allosteric conformational transition[9].

By correlating this information, it seems reasonable to propose that the spectral changes we have reported are themselves an indication of the pH-induced structural transition which, according to the simple model outlined above, is expected to occur in liganded Hb trout IV. This being the case, we may be provided with a method to test possible correlations between the spin state of the molecule and its quaternary structure, and to follow the dynamics of the allosteric transition in Hb trout IV. Experiments along these lines are in progress.

We thank Mr S. Polzoni for this help in preparing the purified Hb components, and R. J. P. Williams for suggesting the possible interpretation of the spectral shift.

BRUNO GIARDINA

FRANCA ASCOLI

MAURIZIO BRUNORI

Laboratory of Molecular Biology,
University of Camerino,
Camerino and
CNR Centre of Molecular Biology,
Institutes of Chemistry and Biochemistry,
Faculty of Medicine,
*University of Rome, Rome, Italy*

[1] Brunori, M. in *Current topics in cellular regulation*, 9 (edit by Horecker, B. L., and Stadman, E R.), 1–39 (Academic, New York and London, 1975)
[2] Binotti, I., et al., *Archs Biochem. Biophys.*, 142, 274–280 (1971)
[3] Brunori, M., et al., *Eur. J. Biochem.*, 39, 563–570 (1973).
[4] Brunori, M., Falcioni, G, and Rotilio, G., *Proc. natn Acad. Sci U.S A.*, 71, 2470–2472 (1974).
[5] Brunori. M., Falcioni. G, Fortuna, G., and Giardina, B., *Archs Biochem Biophys*, 168, 512–519 (1975)
[6] Gray, R. D., and Gibson, Q H., *J. biol Chem* 246, 7168–7174 (1971)
[7] Barra, D., Bossa, F. Bonaventura, J, and Brunori, M., *FEBS Lett.*, 35, 151–154 (1973)
[8] Giardina, B, Brunori, M., Binotti, I, Giovenco, S, and Antonini, E., *Eur. J. Biochem*, 39, 571–579 (1973).
[9] Perutz, M. F, Ladner, J E., Simon, S R, and Ho, C., *Biochemistry*, 13, 2163–2173 (1974).

Corrigendum

In the article "Domoic and quisqualic acids as potent amino acid excitants of frog and rat spinal neurones" by T. J. Biscoe, R. H. Evans, P. M. Headley, M Martin and J. C. Watkins (*Nature*, 255, 166–167; 1975) the structural formula given for quisqualic acid in Fig. 1*d* and Table 2 is incorrect! The structure proposed by Takemoto et al. (Takemoto, T., Nakajima, T., Arihara, S., and Koike, K., *Yakagaku Zasshi*, 95, 326–322 (1975) and 95, 448–452 (1975) is reproduced below

In paragraph 2, line 11, the word isoxazolidinedione should read dioxo-oxadiazolidine.
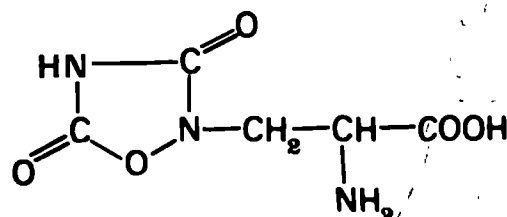
reviews

In the diversity of today's astrophysics, where does radio astronomy stand? That question arises when reading the book by the staff of the National Radio Astronomy Observatory (NRAO) in the United States, a book whose title aims without restriction at the entire Universe beyond the Solar System. One would expect it, then, to cover the vast terrain of astrophysical interactions and phenomena, as seen in the radio window's perspective. A survey of the approximately 1,000 entries in the subject index confirms that indeed an immense variety of objects and processes are touched upon. There are 12 chapters each by a single author; the only chapter on technique *per se*, on "Interferometry and Aperture Synthesis", was written by a pair of authors, synthesising expertise nurtured at Caltech and Cambridge, respectively. It is a wise choice of the editors and a valuable contribution by these authors, providing so clear an exposition of what is the outstanding observation technique at radio wavelengths whenever imaging elements smaller than one arc minute, positional accuracy better than one arc second, and sensitivities per beam lower than a few milli Jansky are called for. Having begun using 1–5-km arrays so successfully at decimetre and centimetre wavelengths, this extremely versatile technique that so thoroughly exploits current computer capacities, is spreading to millimetre-wavelength astronomy and to transcontinental interferometry. The chapter on observation (and reduction) technique conveniently separates the first nine

Bridging the gap in radio astronomy

H. van der Laan

Galactic and Extra-Galactic Radio Astronomy. By the Staff of the National Radio Astronomy Observatory. Edited by G. L. Verschuur and K. I. Kellermann. Pp.x+402. (Springer: Berlin and New York, 1974.) DM 98.30; $40.10.

chapters that deal with galactic subjects, from the last three, which explore extragalactic objects.

The book more than spans the gap that exists between current textbooks and current reviews in radioastronomy. (Of the former there are, because of the swift developments in all fields of astronomy, only one or two worth buying; the latter are, however, numerous, both under auspices of the International Astronomical Union and in several annual and summer school series.) There is no editorial uniformity in the treatment of first principles or the completeness with which current insights are presented. The editors seem to have limited their rule to the choice and sequence of chapters and authors, and thus achieve coherence without blurring their authors' diversity. Some of the chapters are authoritative reviews,

others unpretentious sketches, and a few are highly personal accounts. All are worth reading, some deserve and require close study. Many chapters, singly or in groups, provide an admirable means of progressing soundly from basic matters to current journal literature; thus they attain a major purpose of all postgraduate courses. Active research astronomers found time to write this excellent book and aspiring researchers will spend their time well in reading from it, to each his own selection.

No completeness is possible on this vast plain of astronomy. Of the omissions only the topic of continuum emission from normal galaxies and their nuclei is regrettable, all the more so since the present director of this same NRAO did the pioneering work. But I regard this book as a fitting closure of radio-astronomy's adolescence. Well nigh every astronomical object and astrophysical process is now touched by this technique and this scope makes it imperative to deal with themes and subjects in a multi-spectral context; this is the case even when discussing objects that are first and foremost radio sources.

Symposia or books confined to radio astronomy will increasingly combine subjects that lack all affinity and separate those which are naturally all of one piece. To acknowledge this means to adapt courses of instruction, conferences and even the structure of organisations such as the International Astronomical Union; it is the recognition that radioastronomy has come of age. □

Now that Magnus Pyke has become a television personality, known to the public for his extraordinary antics which somehow succeed in explaining scientific principles, a new book bearing his name might be expected to use the same type of technique. Those who buy *Success in Nutrition* expecting a good laugh combined with a little painless and easily assimilated knowledge are likely to be disappointed. This book, although well-written and easy to read, is a straightforward and orthodox textbook, aimed at 'O' level and 'A' level students of food and nutrition, 'A' level students in home economics and those taking OND and HND courses in catering and

hotel management. It is also recommended for the serious reader following a self-study course. As a

Pyke's guide to nutrition

Success in Nutrition. By Magnus Pyke. Pp. x+227. (John Murray: London, 1975.) £1.95.

textbook it is excellent. All the various constituents of our diet are described, details being given of their occurrence in different foods,

and the effects of cooking and preparing them on the different ingredients. Dr Pyke is always careful to point out gaps in our knowledge, and to ridicule the absurd statements produced by the faddist and the crank. Some readers may think that he is overgenerous in his commendation of the food-processing industry. The only gap in the text is the omission of any statement about 'roughage'. There seems little doubt that civilised people may suffer from the lack of fibre in their food, and that its replacement relieves constipation and many more serious complaints. I would have been interested in Dr Pyke's comments. **Kenneth Mellanby**

Particles in the sea

Suspended Solids in Water. (Marine Sciences, vol. 4.) Edited by Ronald J. Gibbs. Pp. xiii+320. (Plenum: New York and London, 1974.) $34.90.

THE pioneering observations by N. G. Jerlov of the light-scattering properties of sea water, obtained during the Swedish Deep-Sea Expedition (1947–48), established their usefulness for delineating the distribution and relative concentrations of suspended particles in the sea. Interest in this topic has been revived fairly recently by advances in several disciplines, including those concerned with the influence of dilute particle suspensions on the stability and mechanism of fine-grained sediment motion, with the widespread occurrence of high concentrations of particles close to the deep-sea floor (the so-called nepheloid layer) and with developments in marine optics, which have produced some novel designs of *in situ* light-scattering and transmission meters. This volume represents the proceedings of a symposium of the same title, held in Santa Barbara, California, during March 1973, at which recent work on these topics was intensively reviewed.

The first section of the book contains an introductory chapter by the editor and a chapter describing a theoretical study of the settling and reaction of particles in natural waters. In Section II, five chapters deal with the requirements of optical instruments for *in situ* use and the problems of measuring certain optical parameters of sea water. This section is a useful summary of the state-of-the-art.

Sections III and IV, occupying the major part of the book, contain case studies of suspended matter in nearshore and offshore environments using, in most cases, a combination of *in situ* light-scattering data and some measure of the total quantity of suspended material in discrete water samples. The interest here has been to use the optical data as a convenient measure of the concentration of suspended particles and then to examine the role of such material in the redistribution and sedimentation of organic and inorganic solids in the sea.

A particularly good example of an integrated approach to this type of problem is provided by Biscaye and Eittreim who describe a time series of observations of the nepheloid layer at two stations in the western North Atlantic, using measurements of excess radon to study the effect of vertical mixing on the distribution of bottom particles.

The last two chapters show that it is possible to identify sources of suspended particles from a knowledge of their chemical and mineralogical composition. This type of information will be required in studies of the generation and dissipation of nepheloid layers and the role these layers might have in the overall sedimentary economy of the deep sea. What seems to be required is a calibration of these techniques by determining the major and minor element composition of suspended material over an entire water column, and not simply the near-bottom layers in isolation. Such information will, I hope, be made available by the GEOSECS programme of the US International Decade of Ocean Exploration.

S. E. Calvert

Behaviour differences

Sex Differences in Behavior. (Seminars in Human Reproduction.) Edited by Richard C. Friedman, Ralph M. Richart and Raymond L. Vande Wiele. Pp. xvi+495. (Wiley: New York and London, December 1974.) £12.50.

THIS book contains 24 chapters by over 30 different authors, based on a conference held in New York in September 1973. The common theme is behavioural sex differences, but many of the chapters have little else in common. A wide variety of different approaches within psychology and biology are represented, so that the subject is considered from physiological, clinical, evolutionary, psychoanalytical, cognitive developmental, ethological, and experimental psychological viewpoints. Such diversity has resulted in a very uneven book, and this is further intensified by there being a mixture of research papers, surveys of an author's own research, and more general reviews.

The most useful chapters are those which present a survey of an author's own research, particularly where this covers several years' work. Such chapters include those by Moss, on human mother–infant interactions, Phoenix, on prenatal hormones and primate behaviour, Ehrhardt and Baker, on foetal androgens and human sex differences, Sackett, on sex differences in responses to maternal deprivation, Rosenblum, on mother–infant attachment in primates, and Green, on boys who preferred a feminine role. All of those chapters will be useful to research workers, and possibly to advanced undergraduates. Other chapters of that type—for example, that by Lewis and Weinraub on sex differences in infant attachment—are marred by the excessive detail the authors present in reporting their own data.

Chapters consisting of critical but selective reviews are also very useful in a book of this sort, and so are reviews which attempt to organise and integrate previous findings in a new way. I found the reviews by Meyer-Bahlberg, on the XYY syndrome, Levine, on sex differences in rats' responses to neonatal stress, Korner, on human neonatal differences, and Whalen, on problems and concepts in research on sexual differentiation, the most useful. Two other reviews, by Moyer, on sex differences in aggression, and Hamburg, on an evolutionary perspective on behavioural sex differences, both conspicuously lacked critical appraisal of their material. In addition, the former merely presented material which will be published elsewhere.

Several other chapters give incomplete accounts of research work in progress, sometimes based on very small samples, and thus meriting at best only a short report in a journal. For example, Michael *et al.* base a chapter on the bisexual behaviour of female monkeys simply on observations from two animals; Kohlberg and Ullian, on the development of gender concepts, report data from two subjects (out of 70 studied); Meyer-Bahlberg *et al.*, on cryptorchidism and gender identity, use results from 10 subjects (out of 67 studied). Perksy's chapter on the menstrual cycle and psychological test measures also uses partly analysed data. The only really successful chapter reporting original experimental work is that of Doering *et al.*, on testosterone and psychological test measures in men, and even that is really more appropriate for a journal.

Overall, the book is very diverse and very uneven, so that at £12.50 it is unlikely to attract many buyers. Parts of it will be useful to a wide variety of people for a wide variety of reasons, and I would anticipate that there will be a heavy demand for library copies.   **John Archer**